

**DEVELOPMENT OF COLOR SENSOR BASED RAPID
METHOD FOR DETECTION OF VEGETABLE OILS
ADULTERATION IN GHEE**



**THESIS SUBMITTED TO THE
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, KARNAL
(DEEMED UNIVERSITY)
IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF**

DOCTOR OF PHILOSOPHY

IN

DAIRY CHEMISTRY

BY

YUGAL KISHOR NAIK

M.Tech (Dairy Chemistry)

**DAIRY CHEMISTRY DIVISION
ICAR- NATIONAL DAIRY RESEARCH INSTITUTE
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KARNAL-132001 (HARYANA), INDIA**

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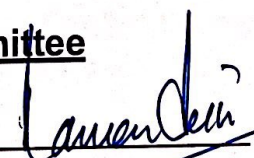
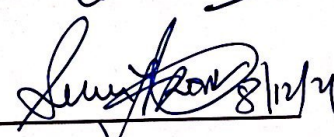
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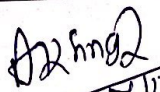

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This is to certify that the thesis entitled “**DEVELOPMENT OF COLOR SENSOR BASED RAPID METHOD FOR DETECTION OF VEGETABLE OILS ADULTERATION IN GHEE**” submitted by **YUGAL KISHOR NAIK** in partial fulfillment of the requirements for the award of the degree of **DOCTOR OF PHILOSOPHY IN DAIRY CHEMISTRY** of the **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE, (DEEMED UNIVERSITY)**, Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance. The work embodied in this thesis is original and no part has been submitted in part or full for the award of any diploma or degree of this or any other university.

Date: 15 / 09 / 2021


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*Dedicated to
Respected Guide
&
My Beloved
Family*

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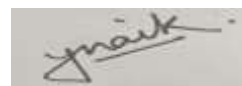
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All are not mentioned but no one is forgotten

. 09. 2021

A rectangular box containing a handwritten signature in dark ink. The signature appears to be 'Yugal Kishor Naik' written in a cursive, stylized script.

Yugal Kishor Naik

Development of color sensor based rapid method for detection of vegetable oils adulteration in ghee

ABSTRACT

Ghee is considered as a premium product in Indian dairy Industry. It is the second largest milk product after the fluid milk. The premium nature of ghee attracts fraudulent producers or middlemen involved in ghee trade to adulterate ghee with vegetable oils. The adulteration is done in such a meticulous manner that adulterated ghee passes the FSSAI specified standards. Ghee standard states that no synthetic antioxidant is permitted in ghee (FSSR, 2011). On the contrary in vegetable oils the synthetic antioxidants like Butylated Hydroxyanisole (BHA), Tertiary Butylhydroquinone (TBHQ) are permissible to the tune of maximum 200 ppm (FSSR, 2011). Therefore, in the present study the possibility of using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Cerium (IV) oxide nanoparticles (CeO_2) to detect these antioxidants and thereby the addition of vegetable oils in ghee was explored. 20 μL of 4mM solution of DPPH was found optimum to bring a perceptible color change in 0.02 - 0.0025% acetonitrile solution of antioxidants i.e., BHA, TBHQ, and ascorbyl palmitate (AsP). The time taken to change the color depended upon the concentration (0.0025-0.02%) of antioxidants in the acetonitrile solution and was found to be 3- 27 min for BHA, 1- 5.8 min for TBHQ and 0.5 – 2 min for AsP. In case of 20% dispersion of CeO_2 20 μL of CeO_2 as well as CeO_2 impregnated dried paper discs did not show a change in color after the addition of antioxidant BHA and TBHQ (0.02, 0.01, 0.005 and 0.0025%) solutions in acetonitrile. However, color change was observed in Gallic Acid (GA), Ascorbic Acid (AA) and AsP by using the same concentration of CeO_2 dispersion and CeO_2 impregnated dried paper discs. DPPH impregnated dried paper-based disc sensors and tube sensors were developed in the study. The disc and tube sensors worked well and their response time was dependent on their concentration (0.0025- 0.02%). The above sensors response time were same as on addition of optimized DPPH solution in the studied concentrations of antioxidant (BHA, TBHQ and AsP) solutions. Double extraction with acetonitrile was found to be more efficient in extracting the antioxidants from ghee samples spiked with antioxidants (BHA, TBHQ and AsP). Using the developed color-based disc sensors, a distinct color change was observed in about 143 min for cow ghee samples spiked with palm oil @ 1% and about 60 min in case of cow ghee samples spiked with sunflower oil. In both the cases the response time was well below 246 min indicating that it was possible to detect addition of vegetable oils at a level of 1% and above. The storage stability of tube and disc sensor determined in terms of their response time to change color in pure solutions was found to be 15 and 30 days, respectively at ambient temperature (30°C) and 30 and 90 days, respectively at refrigerated temperature ($4-6^\circ\text{C}$). Column SPB-5(30m x 0.53 mm x 1.5 μm) and the standardized GLC conditions resulted efficient clear resolution of all the three synthetic antioxidants i.e., BHA, TBHQ and BHT. The retention time observed for BHA, BHT and TBHQ were 7.45, 8.0 and 8.35 min, respectively. The percent recovery of TBHQ and BHA observed in ghee was 97.73 ± 4.98 and 97.59 ± 5.38 , respectively. As a result of storage, peroxide and TBA values of all ghee samples spiked with vegetable oils showed an increase and led to a corresponding increase in the response time of the paper-based disc sensors to change the color. It was observed that in case of ghee samples spiked with palm oil @1% and subsequently stored, the response time came close to 240 min after 5 months of storage. However, in case of cow ghee samples spiked with sunflower oil and subsequently stored, the response time of the paper-based disc sensor was very much below the time of 246 min even after 9 months of storage. Developed dye impregnated paper-based disc sensors were evaluated in market ghee samples (6 branded and 6 unbranded). It was found that 4 samples out of the 6 in each case showed the response time to be in the range of 1- 12 min, which was well below the time of 246 min which indicated the presence of antioxidants. GLC analysis of the corresponding positive samples also showed the presence of a peak corresponding to the retention time of TBHQ. Reversed- phase thin layer chromatography (RP-TLC) analysis of branded samples showed the presence of β - sitosterol in 2 samples, whereas, in case of unbranded samples the β - sitosterol appeared in 3 samples. This indicated that not only the vegetable oils but synthetic antioxidants are also being added in ghee samples available in the market.

घी में वनस्पति तेल की मिलावट की जाँच हेतु रंग संवेदक आधारित

तीव्र विधि का विकास सारांश

भारतीय डेयरी उद्योग में घी को एक प्रीमियम उत्पाद माना जाता है। यह द्रव दूध के बाद दूसरा सबसे बड़ा दुग्ध उत्पाद है। घी की प्रीमियम प्रकृति मिलावटी उत्पादकों या घी के व्यापार में शामिल बिचौलियों को वनस्पति तेलों के साथ घी में मिलावट करने के लिए आकर्षित करती है। मिलावट इतनी सावधानी से की जाती है कि मिलावटी घी एफएसएसएआई द्वारा निर्दिष्ट मानकों को पार कर जाता है। घी मानक कहता है कि घी में किसी भी सिंथेटिक एंटीऑक्सीडेंट की अनुमति नहीं है (एफएसएसआर, 2011)। इसके विपरीत वनस्पति तेलों में सिंथेटिक एंटीऑक्सीडेंट जैसे ब्यूटाइलेटेड हाइड्रोक्सीनिसोल (बीएचए), तृतीयक ब्यूटाइलहाइड्रोक्विनोन (टीबीएचक्यू) की अधिकतम 200 पीपीएम (एफएसएसआर, 2011) की अनुमति है। इसलिए, वर्तमान अध्ययन में इन एंटीऑक्सीडेंट का पता लगाने के लिए 2,2-डाइफिनाइल-1-पिक्रिलहाइड्राइल (DPPH) और सेरियम (IV) ऑक्साइड डिस्पर्सन (CeO_2) का उपयोग करके पता लगाया गया था और इस तरह घी में वनस्पति तेलों को मिलाने की संभावना का पता लगाया गया था। DPPH के 4mM घोल के 20 μL को एंटीऑक्सीडेंट यानी BHA, TBHQ, और एस्कोर्बिल पामिटेट (AsP) के 0.02 - 0.0025% एसीटोनिट्राइल घोल में एक बोधगम्य रंग परिवर्तन लाने के लिए इष्टतम पाया गया। रंग बदलने में लगने वाला समय एसीटोनिट्राइल घोल में एंटीऑक्सीडेंट की सांद्रता (0.0025- 0.02%) पर निर्भर करता है और बीएचए के लिए 3-27 मिनट, टीबीएचक्यू के लिए 1-5.8 मिनट और एसपी के लिए 0.5 - 2 मिनट पाया गया। CeO_2 के 20% डिस्पर्सन को CeO_2 के 20 μL के साथ-साथ CeO_2 संसेचित सूखे पेपर डिस्क को एसीटोनिट्राइल में बनाये हुए एंटीऑक्सीडेंट बीएचए और टीबीएचक्यू (0.02, 0.01, 0.005 और 0.0025%) विलयन को डालने के बाद रंग में बदलाव नहीं दिखाया। हालांकि, CeO_2 डिस्पर्सन और CeO_2 संसेचित सूखे पेपर डिस्क की समान सांद्रता का उपयोग करके गैलिक एसिड (GA), एस्कोर्बिक एसिड (AA) और AsP में रंग परिवर्तन देखा गया था। अध्ययन में डीपीपीएच के सूखे कागज आधारित डिस्क सेंसर और ट्यूब सेंसर विकसित किए। डिस्क और ट्यूब सेंसर ने अच्छी तरह से काम किया और उनका प्रतिक्रिया समय उनकी सांद्रता (0.0025- 0.02%) पर निर्भर था। उपरोक्त सेंसर के प्रतिक्रिया समय एंटीऑक्सीडेंट (बीएचए, टीबीएचक्यू और एसपी) विलयन के अध्ययन किए गए सांद्रता में अनुकूलित डीपीपीएच विलयन के समान था। एसीटोनिट्राइल के साथ डबल निष्कर्षण को एंटीऑक्सीडेंट (बीएचए, टीबीएचक्यू और एसपी) के साथ घी के नमूनों से एंटीऑक्सीडेंट निकालने में अधिक कुशल पाया गया। विकसित रंग-आधारित डिस्क सेंसर का उपयोग करते हुए, गाय के घी के नमूनों के लिए लगभग 143 मिनट में ताड़ के तेल @ 1% और गाय के घी के नमूनों में सूरजमुखी के तेल के साथ लगभग 60 मिनट के लिए एक अलग रंग परिवर्तन देखा गया। दोनों ही मामलों में प्रतिक्रिया समय 246 मिनट से काफी कम था जो दर्शाता है कि 1% और उससे अधिक के स्तर पर वनस्पति तेलों की मिलावट का पता लगाना संभव था। शुद्ध घोल में रंग बदलने के लिए उनके प्रतिक्रिया समय के संदर्भ में निर्धारित ट्यूब और डिस्क सेंसर की भंडारण स्थिरता क्रमशः परिवेश के तापमान (30°C) पर 15 और 30 दिन एवं प्रशीतित तापमान (4-6°C) पर 30 और 90 दिन पाई गई। कॉलम SPB-5 (30m x 0.53 mm x 1.5 μm) और मानकीकृत GLC स्थितियों के परिणामस्वरूप सभी तीन सिंथेटिक एंटीऑक्सीडेंट यानी BHA, TBHQ और BHT का कुशल स्पष्ट समाधान हुआ। बीएचए, बीएचटी और टीबीएचक्यू के लिए अवधारण समय क्रमशः 7.45, 8.0 और 8.35 मिनट था। घी में देखे गए टीबीएचक्यू और बीएचए की प्रतिशत वसूली क्रमशः 97.73 ± 4.98 और 97.59 ± 5.38 थी। भंडारण के परिणामस्वरूप, वनस्पति तेलों के साथ सभी घी के नमूनों के पेरॉक्साइड और टीबीए मूल्यों में वृद्धि देखी गई और रंग बदलने के लिए कागज-आधारित डिस्क सेंसर के प्रतिक्रिया समय में एक समान वृद्धि हुई। यह देखा गया कि घी के नमूनों में ताड़ के तेल @ 1% के साथ मिलावट करके संग्रहीत किया गया, प्रतिक्रिया समय 5 महीने के भंडारण के बाद 240 मिनट के करीब आ गया। हालांकि, गाय के घी के नमूनों में सूरजमुखी के तेल के साथ मिलावट करके संग्रहीत किया गया, कागज आधारित डिस्क सेंसर का प्रतिक्रिया समय 9 महीने के भंडारण के बाद भी 246 मिनट के समय से बहुत कम था। विकसित डाई इंप्रेग्नेटेड पेपर-आधारित डिस्क सेंसर का मूल्यांकन बाजार के घी के नमूनों (6 ब्रांडेड और 6 अनब्रांडेड) में किया गया था। यह पाया गया कि प्रत्येक मामले में 6 में से 4 नमूनों ने प्रतिक्रिया समय 1-12 मिनट की सीमा में दिखाया, जो 246 मिनट के समय से काफी कम था जो एंटीऑक्सीडेंट की उपस्थिति का संकेत देता था। संबंधित सकारात्मक नमूनों के जीएलसी विश्लेषण ने टीबीएचक्यू के अवधारण समय के अनुरूप शिखर की उपस्थिति को भी दिखाया। ब्रांडेड नमूनों के उल्टे-चरण पतली परत क्रोमैटोग्राफी (आरपी-टीएलसी) विश्लेषण ने 2 नमूनों में β -साइटोस्टेरोल की उपस्थिति को दिखाया, जबकि अनब्रांडेड नमूनों के मामले में 3 नमूनों में β -साइटोस्टेरोल दिखाई दिया। इससे संकेत मिला कि बाजार में उपलब्ध घी के नमूनों में न केवल वनस्पति तेल बल्कि सिंथेटिक एंटीऑक्सीडेंट भी मिलाए जा रहे हैं।

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LIST OF ABBREVIATIONS AND SYMBOLS

IMARC	International Market Analysis Research and Consulting Group
AOAC	Association of Official Analytical Chemists
BIS	Bureau of Indian Standards
FSSAI	Food Safety and Standard Act of India
AR	Analytical Reagent
GLC	Gas Liquid Chromatography
RP-TLC	Reverse phase- thin layer chromatography
LR	Laboratory Reagent
SD	Standard Deviation
RSD	Relative Standard Deviation
USM	Unsaponifiable matter
ISI	Indian Standards Institution
ICAR	Indian council of agricultural research
TBA	Thio-barbituric acid
TCA	Trichloro acetic acid
DPPH	2, 2 diphenyl-1-picrylhydrazyl
BHA	Butylate hydroxy anisole
BHT	Butylate hydroxy toluene
TBHQ	Tert-butylated hydro quinone
GA	Gallic acid
AA	Ascorbic acid
AsP	Ascorbyl palmitate
PO	Palm oil
SFO	Sunflower oil
μV	Micro volt
Sl no	Serial number
%	Percent
<	Less than
>	Greater than
$^{\circ}\text{C}$	Degree Celcius
μ	Micron

B	Beta
μL	Microlitre
w.r.t	With respect to
Ppm	Parts per million
Cm	Centimeter
G	Gram
Hr	Hour
Kg	Kilogram
lt	Litre
Mg	Milligram
Min	Minute
Mm	Millimetre
Nm	Nano meter
ml	Millilitre
mM	Milimolar
M	Molar
N	Normal
RPM	Revolution per minute
Sec	Second
v/v	Volume/volume
w/v	Weight/volume
w/w	Weight/weight

CHAPTER - 1

Introduction

Introduction

Ghee has been used in India since 1500 BC (Achaya, 1997). It is the second largest dairy product after fluid milk. It is reported that about 35% of the milk is converted into ghee in Indian sub-continent (Hazra *et al.*, 2017). The market of ghee in India was valued at INR 2374 billion in 2020 (IMARC, 2020).

Ghee is rich in fat soluble vitamins (A, D, E and K) and a few of the essential fatty acids such as conjugated linoleic acid (CLA), linolenic acid and arachidonic acid. CLA has been reported to decrease the risk of cancer, diabetes, atherosclerosis and body fat mass (Belury, 2002). McBrain *et al.* (1997) showed induced apoptotic cell death to demonstrate the anticarcinogenic properties of butyric acid present in ghee. It is a premium product because of its high nutritional value, pleasant aroma and high calorific value.

Gonzalez *et al.* (2010) has defined adulteration as the process by which the quality or the nature of a given substance is reduced through the addition of a foreign or an inferior substance and the removal of vital elements. The fraudulent producers or middlemen involved in ghee trade tend to adulterate the milk fat with vegetable oils like palm oil, sunflower oil, soybean oil etc. The adulteration is done in such a meticulous manner that adulterated ghee passes the FSSAI specified standards.

In recent years, the problem of adulteration of vegetable oils in ghee has assumed a very serious dimension. Such a situation has tarnished the image of the dairy industry, not only in India, but also in the overseas markets. In order to ensure a quality product to the consumer, the Government of India has specified the quality standards for ghee under FSSAI (2011) and Agmark standards (1938). These standards were relevant in the past but over the years the relevance of these standards has been reduced. The reason of this change may be attributed to first, the wide variations in the physico-chemical makeup of milk fat owing to many factors like animal species, feeding habits, etc. and second, the meticulous adulteration malpractices followed by the unscrupulous traders.

It is well established that commercially available refined edible vegetable oils have low shelf life due to the unsaturation and thus synthetic antioxidants or their mixtures are added to improve their oxidative stability (O'brien, 2008).

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 FSSAI guidelines.

Several conventional methods like High performance liquid chromatography (HPLC), Gas-Liquid Chromatography (GLC) and Reversed-phase thin layer chromatography (RP-TLC) used for the detection of vegetable oils (palm oil, soyabean oil, coconut oil and sunflower oil etc.) adulteration in ghee are complex, time consuming and costly. Physico-chemical parameter-based methodologies like Reichert-meissl value, polenske, iodine value, saponification value and butyro-refractometer reading, crystallization time test and opacity test etc. reported so far have their own limitations in detecting the type and limit of detection. Already reported rapid methods like E-nose and FT-NIR used to detect ghee/ milk fat adulteration are costly because of sophisticated instruments involved. Some rapid chemical based methods like modified Bieber's test and chromogenic test have reported limit of detection 7.5% and 5%, respectively. Hence, there was a need to develop a simple, more stable and ready-to-use color sensor based rapid method with low limit of detection. Therefore, the present project was proposed with the following objectives:

- 1. Development of color sensor for synthetic antioxidant detection**
- 2. Storage stability of developed rapid color-based sensors**
- 3. Evaluation of developed rapid color-based sensor (Tube/Card/Disc) in stored and market ghee sample**

CHAPTER - 2

Review of Literature

Review of Literature

“Ghee” means the pure clarified fat derived solely from milk or from desi (cooking) butter or from cream to which no coloring matter or preservative has been added (FSSR, 2011). Milk fat is considered as the most important constituent of dairy products. It imparts typical body and aroma to milk and its related products. It is also a major determinant in the costing of milk and its products. Major part of milk fat consists of triglycerides. Minor constituents of milk fat include mono- and di- glycerides, phospholipids, fat soluble vitamins, cholesterol, squalene, waxes, etc. Milk fat is frequently consumed by Indian peoples in the form of ghee. As stated above, the premium nature of milk fat and its variable composition attracts the unscrupulous traders to adulterate this fat with other fats of cheaper value. The most common and prominent adulterants are vegetable fat in the form of oils such as palm oil, sunflower oil, soybean oil etc. Widespread study of the literature revealed that several methods e.g., physico- chemical constant based, Chromatographic methods, Spectroscopic methods and some trace component-based methods, have been developed to detect the adulteration of vegetable oils in ghee. The literature is reviewed under the following four major heads:

2.1 Physico-chemical constant-based methodology:

Certain well-known physico-chemical constants have been specified in the standards of ghee (FSSR, 2011) to warrant the better quality of ghee. These parameters include the BR Reading at 40°C, Reichert-Meissl value, the polenske value. Iodine value which measures the degree of unsaturation and the saponification value which gives some clue as to the average size of fatty acids in a given sample of fat has also been mentioned to check the purity of milk fat (Rangappa and Achaya, 1974). These physico-chemical constants are elaborated briefly in the following different sections:

2.1.1 Reichert-Meissl value:

In milk fat RM value varies between 17 - 35, which is mostly higher value (generally 1) for all other different fats and oils the exception oils are coconut and palm kernel for which its value varies between 4-8 (Singhal, 1980). Kumar (2008) reported that adulteration of cow ghee with

individual vegetable oils and animal body fats was detectable at 10% level, whereas for buffalo ghee it was possible to detect at 15% level of adulteration.

Upadhyay (2014) reported that groundnut oil when added singly in cow ghee at as low as 10% level could be detected, however pooling of buffalo and cow ghee revealed that groundnut oil detection could be done at 15% and above using R.M. value as a parameter of quality.

Gandhi *et al.* (2014) reported that sheep body fat and palmolein adulterated individually at 15% levels could be detected in pooled samples of buffalo and cow ghee based on determination of RM value. Mixture of sheep body fat and palmolein (9:21 & 6:14) was detectable at 30% & 20 % levels, respectively.

Ahmed *et al.* (2020) reported that hydrogenated vegetable oils, tallow, coconut oil and soyabean oil adulterated individually at 5% or more could be detected in ghee samples by using RM value determination.

Yadav (2020) developed a wash RM protocol for RM adjusted palm oil detection in buffalo and cow ghee. The pure buffalo and cow ghee adulterated with RM adjusted palm oil (32) @ of 2.5, 5.0, 7.5 and 10.0 %. They concluded that wash RM- protocol for determining the RM- value adjusted ghee could be detected at minimum 2.5 % level.

Chaudhari *et al.* (2021) reported that adulteration of palm oil in buffalo and cow ghee separately at minimum 5% levels could be detected based on RM value determination.

2.1.2 Polenske value:

For milk fat the polenske value varies between 1.2 to 2.4. This value for other oils and fats (Singhal, 1980; Winton and Winton, 1999) is also low (less than 1) except for the palm kernel oil (6-12) and coconut oil (15-20).

Kumar (2008) reported that the adulteration of pure cow & buffalo ghee with vegetable oils could not be detected even at 20% level, except adulteration of buffalo ghee with the palm and soybean oils which could be detected at 15% level by determination of Polenske value. Patel (2011) concluded that the adulteration of Soybean oil and palm oil in buffalo ghee at 15% level could be detected by using Polenske value as a quality parameter.

Upadhyay (2014) reported that the polenske value did not help in the detection of groundnut oil adulterated to cow ghee and pooled sample of buffalo and cow ghee at any of the levels (5,10 and 15%).

2.1.3 Refractive index and Butyrorefractometer reading:

This property determines the degree of bending of light waves passing over a liquid or transparent solid. This bending is a representative of the particular solid or liquid. For oils and fats, both refractive index and butyrorefractometer reading increases with increase in chain length and unsaturation of the fatty acids. For most of the vegetable oils, it ranges varies from 1.4600 to 1.4750 and for some of the vegetable oils like lauric fats (coconut & palm kernel oils) and palm oil, where it lies in the ranges between 1.4480 to 1.4580 (Hamilton and Rossell, 1986; Karleskind, 1996). The refractive index of milk fat varies between 1.4538 to 1.4578 (Jenness and Patton, 1969).

The B.R. reading of milk fat ranged from 40-45 and vegetable oils and fats (above 50), which are e so wide apart (Singhal, 1980; Gunstone *et al.*, 1994), hence this property could be safely employed for detection of vegetable oils adulteration in milk fat, except coconut oil (38-39) and palm oil (39-40) where feeding of cottonseed oil leads to raise the B.R. reading by 5 units in case of ghee (Rangappa and Achaya, 1974).

Arora *et al.* (1996) developed a platform test for mustard oil detection in milk at a minimum level at 10% of higher level. Kumar (2003) reported that vegetable oil adulteration up to 5% in cow ghee and 15% in buffalo ghee could be detected. Patel (2011) reported that the vegetable oil adulteration in ghee up to a 10% level could easily be detected by BR- reading.

Adulteration of pure ghee either buffalo or cow with coconut oil at 25% level or above could be detected based on RI determination (Salem *et al.*, 2019).

Nimbkar and Shanmugasundaram (2019) reported that the adulteration of soyabean oil to ghee up to a 20% level could easily be detected by BR.

Chaudhari *et al.* (2021) reported that the palm oil adulteration in buffalo and cow ghee separately at minimum 10% levels could be detected based on BR value determination.

2.1.4 Iodine value:

Number of grams of iodine absorbed by 100g of fat under specified conditions is the iodine value. It is a measure of unsaturated linkages present in a fat. For milk fat its value varies between 26-35, which is low when compared to most of the other oils and fats (Singhal, 1973). For vegetable oils, the value is very high (74-145) except palm kernel oil (10-18) and coconut oil (6-10) .

Upadhyay (2014) reported that adulteration of groundnut oil at 5% level could be detected, when added singly in cow ghee, while in buffalo ghee, the detection level was 10% and above using iodine value. Adulteration of pure ghee either buffalo or cow coconut oil at 25% level could be detected (Salem *et al.*, 2019).

2.1.5 Saponification value:

Saponification value which denotes the number of milligrams of KOH required to saponify 1 g of fat gives an indication of average molecular weight of fatty acids present. For milk fat and vegetable oils, the value ranges from 210 to 233 and 170 to 197, respectively. Palm kernel oil and coconut oil show higher saponification value ranging from 243 to 262 (Jenness and Patton, 1969; Singhal, 1980).

Several earlier workers (Achaya and Banerjee, 1946; Ali and Tremazi, 1966; Velu, 1971) have reported that physico-chemical constants failed to detect the milk fat adulteration with beef tallow, refined cottonseed oil, hydrogenated oils and even coconut oil separately or in mixture even up to 10% level. Kumar (2008) reported that adulteration of vegetable oils in pure cow ghee at 10% levels could be detected by using saponification value as a physico-chemical parameters. In case of buffalo ghee, only 15% level of adulteration with vegetable oils (except palm oil) could be detected. Adulteration of pure ghee either buffalo or cow ghee with coconut oil at minimum level of 25% could be detected (Salem *et al.*, 2019). Nimbkar and Shanmugasundaram (2019) reported that the adulteration of soyabean oil in ghee up to 20% level could easily be detected.

2.1.6 Apparent Solidification Time (AST) Test:

This test was developed by Kumar *et al.*, (2009) to detect the adulteration of vegetable oils in milk fat. The average AST values for buffalo and cow milk fat were 2 min 40 sec and 3 min

10 sec, respectively. Addition of vegetable oils caused an increase in the AST values of pure buffalo and cow milk fat, whereas the addition of vanaspati (hydrogenated vegetable oils) resulted in the decrease in the AST values of buffalo and cow pure milk fat. Adulteration of buffalo milk fat with vanaspati (hydrogenated vegetable fat) was detectable at levels $\geq 10\%$, but not in cow milk fat. Patel *et al.* (2011) reported that the mixture of vegetable oil and buffalo body fat (10:6:5) at 10% level in cow ghee and same mixture (10:15) at 5% level in buffalo ghee could be detected. Gandhi (2018) used this test to solid fraction of milk fat obtained at 15 °C (S15) after solvent fractionation and indicated that addition of a mixture of palm olein and sheep body fat (1:14) in milk fat at a level of 20% could be detected. Nimbkar and Shanmugasundaram (2019) reported that the addition of soyabean oil to ghee up to 20% level could easily be detected by using the above test.

2.1.7 Complete Liquefaction Time (CLT) Test:

The CLT is defined as the time taken by the solid fat to melt completely at a temperature of 44°C. At 44°C, the CLT values of pure cow ghee samples ranged from 2 min 12 sec to 3 min 15 sec, while for pure buffalo ghee samples ranged from 2 min 35 sec to 3 min 15 sec. Addition of vegetable oils (palm, rice bran, and soybean) caused a decrease in the CLT values in both pure cow and buffalo ghee at 44°C. This decrease or increase in CLT values depended upon the number of adulterants added. Higher the quantity of adulterant added, greater the CLT values.

Kumar (2013) had reported that soybean oil could be detected at a level of 15% in cow ghee. Upadhyay (2014) reported that groundnut oil could be detected at a level of 15% in cow ghee while in case of buffalo ghee detection was not possible at the same level and also in the case of pooled buffalo and cow ghee. Upadhyay (2017) concluded that adulteration in milk fat with goat body fat and groundnut oil could be detected at minimum 10% level, either singly or in combination.

Gandhi *et al.* (2018) reported that the adulteration of cow ghee with palm olein could not be detected at any of the level studied, other than at level of 15%. On another side, in cow as well as in buffalo ghee samples separately adulterated with sheep body fat could easily be detected at all the levels studied except 5 % level. The mixture of palm olein and sheep body fat was detected at 6+14 (20) % and 9+21 (30) % level, but not at 3+7 (10) % level. After fractionation, the lower level of 3+7 (10) % was also found to be detectable.

2.1.8 Crystallization Time Test:

The Crystallization time test is defined as the time taken for the milk fat to crystallize at 17°C when dissolved in a solvent mixture of acetone and benzene (3.5:1.0). It is an excellent method to detect the animal body fat.

Kumar (2008) used this test & concluded that crystallization time of ghee samples increased when the samples were adulterated with vegetable oils (palm, rice bran, and soybean). This increase was dependent upon the level of adulteration with vegetable oils. Crystallization time for the pure cow ghee and pure buffalo ghee samples ranged from 6 min 50 sec to 16 min 20 sec and from 6 min 30 sec to 12 min 30 sec with the mean of 11 min 4 sec and 8 min 42 sec, respectively.

Zachariah *et al.* (2010) used this test & reported that cow ghee adulterated with 5% coconut oil showed fair crystallization even after 35 min of incubation at 17°C. But in case of palm oil, limit of detection was 10% as the ghee samples adulterated with 5% palm oil showed crystallization after 20 min. Patel *et al.* (2011) reported that the mixture of vegetable oil and buffalo body fat (10:6.5) could be detected at the level of 75% in cow ghee.

2.1.9 Differential Scanning Calorimetry

Nurrulhidayah *et al.* (2015) reported that the adulteration of butter could be detected through DSC by changes in the cooling and heating curves when adulterated with lard at minimum 1% level. DSC in combination with multivariate statistical analysis technique has also been employed for the detection of adulteration of milk fat with palm oil (Tomaszewska-Gras, 2016)

Upadhyay (2017) exploited this technique for the detection of caprine body fat and groundnut oil in milk fat (cow as well as buffalo) either individually or mixture thereof as there was difference in crystallizing and melting curves of pure milk fat and adulterant. In adulterated milk fat and pure milk fat, although, the total number of crystallizing and melting peaks were same, difference in cooling and melting curves of pure milk fat and adulterated fat were significantly different in mid-range temperature enabling detection of adulterants in milk fat. Further, shapes of endothermic peaks of pure and adulterated milk fat were also different and can be used for detection of adulteration at more than 5% level.

2.1.10 Opacity Test:

Opacity test was developed by Singhal (1980) to detect the adulteration of ghee with tallow and lard based on the time taken by the melted fat sample (5.0 g) to become opaque at 23°C using 590 nm (yellow) filter. Sharma and Singhal (1996) carried out the above test at 25°C for ghee, body fats and vanaspati and ghee samples adulterated @ (5, 10 and 20) % levels and observed the nearly same results as earlier reported by Singhal (1980). Their study concluded that ghee took 35 min or more to become opaque, whereas vanaspati and pig body fat took only 2- 2.5 min and 6- 7 min, respectively. On another hand, goat body fats and buffalo both took 18 min to become opaque. They would not able to detect significant difference in the opacity time of pure ghee and vanaspati and lard adulterated ghee up to 20% level.

Panda and Bindal (1998) modified the procedure of Singhal (1980) and reported that the opacity time is the time required by a fat sample (23°C) to acquire the O.D. in the range of 0.14 to 0.16 and consequent transmittance of 68 to 72 showing the onset of solidification of fat. The opacity time of pure ghee (14-15 min) was much lower than that of ghee adulterated with vegetable oils (21-25 min at 10% level and 19-21 min at 5% level of adulteration).

2.1.11 Critical Temperature of Dissolution (CTD):

Critical temperature of dissolution is the temperature at which turbidity appears on gradual cooling of the fat dissolved in a warm solvent or solvent mixture and it is a characteristic of a particular fat which depends upon the nature of the solvent, nature and amount of most insoluble glycerides (usually trisaturated glycerides) present in a fat as well as the mutual solubilizing power exerted on these glycerides by the soluble glycerides (Rangappa and Achaya, 1974; Boghra *et al.*, 1981). Bhide and Kane (1952) observed the CTD values for vanaspati and ghee in the range of 62 to 72°C and 39 to 45°C and, respectively, using a 2:1 (v/v) mixture of 95% ethanol and iso-amyl alcohol, and also concluded that gross adulteration of vanaspati in ghee could be detected very easily. Delforno (1964) reported the CTD values for coconut oil as 28 to 41°C (average 35°C) and for other oils and fats as 67 to 82°C (average 75°C). The CTD test seems to be simple, but its efficacy is greatly affected by the free acidity (FFA) and rancidity (peroxides). Kumar (2003), using the solvent mixture (ethyl alcohol and iso-amyl alcohol, 2:1), reported that adulteration of ghee up to 15% level with vegetable oils could not be detected by CTD value. Kumar (2013) & Upadhyay (2014) reported that CTD value (degree centigrade) for

pure cow ghee varies between 51.6-54.6 with an average value of 53.3, whereas in case of pure buffalo ghee its value varies between 52.4-56.2 with an average value of 54.3.

Kumar (2013) reported that adulteration of cow and buffalo ghee with soybean oil and buffalo body fat individually at (5, 10 and 15) % levels and in their combinations at 5+5 (10), 10+10 (20) and 15+15 (30) % levels raise the CTD value and this increase was only dependent upon the quantity of added adulterants to the pure cow and buffalo ghee. The level of adulterant increases, was directly increase the CTD value of ghee samples. He was also reported that addition of buffalo body fat slightly more increases the CTD value of ghee as compared to soybean oil. Upadhyay (2014) also reported that using CTD value could be detected only at 15% level of adulteration.

2.2 Chromatographic methods:

2.2.1 Gas-Liquid Chromatography (GLC):

2.2.1.1 Fatty acids analysis:

GLC technique was employed by several workers for the detection of adulterant oils in milk fat. Singhal (1987) reported that the ratio of fatty acids, viz., $C_{15:0}/C_{17:0}$ and $C_{16:0}/C_{18:0}$ was found to be lower in ghee samples adulterated with animal body fats even in the presence of vegetable oils. Kumar (2003) found that the adulteration of ghee with 5% vegetable oils (sunflower oil, soybean oil and groundnut oil) in ghee could be detected easily, using fatty acid ratios such as $C_{14:0}$ to $C_{18:2}$, $C_{16:0}$ to $C_{18:2}$, $C_{18:0}$ to $C_{18:2}$, as well as linoleic acid ($C_{18:2}$) as a marker. Adulteration of ghee with groundnut oil could be detected at a level of $\geq 5\%$ in both cow ghee and buffalo ghee by considering the level of linolenic acid ($C_{18:2}$) and the ratio of some of the fatty acid from $C_{4:0}$ till $C_{10:0}/C_{18:2}$. The same ration could also help in detection of adulteration of mixture of groundnut oil and goat body fat (3:7) at the lowest level of 10 % in both cow and buffalo ghee (Upadhyay, 2014).

Upadhyay *et al.* (2018) reported for the detection of adulterant like groundnut oil and goat body fat in cow ghee and buffalo ghee by utilizing the differences of the fatty acid composition coupled with the Principal Component Analysis (PCA). The concluded that the ghee when added with groundnut oil individually and in combination with goat body fat could be detected at 10%

level, while goat body fat added individually could be detected at minimum 5% level using PCA analysis.

Salem *et al.* (2019) reported that adulteration of pure milk fat, either cow or buffalo with coconut oil up to 25 % level could be detected easily by increase in total short chain (TSC)/total long chain (TLC)% and decrease the percentage of USFA/SFA. It may be due to the addition of coconut oil in milk fat which increased the concentrations of the TSC and SFA fatty acids while diluted the concentrations of the TLC and USFA fatty acids.

2.2.1.2 Triglycerides analysis:

Kuksis and McCarthy (1964) used Gas chromatography for the separation and quantitative estimation of triglycerides by carbon number from native and adulterated butter fats. When the triglyceride type composition of the native butter was known, adulteration with vegetable fat could be detected at the 1% level, as these adulterants added significantly to only a few of the butterfat triglyceride peaks resulting in a noticeable distortion of the chromatographic elution pattern of this fat. Parodi (1973) carried out GLC analysis of butterfat adulterated at various levels with vegetable oils (5% level) was detectable. Luf *et al.*, 1987 also detected 5 to 10% of vegetable oils in butter based on $C_{52:0}/C_{40:0}$ and $C_{50:0}-C_{54:0}/C_{38:0}-C_{40:0}$ triglycerides respectively.

Kala (2013) reported that the addition of beef tallow and coconut oil at a level of 2%, in case of vegetable fat and lard addition could be detected at 5% and 6.3% respectively. Rakesh (2016) reported that the adulteration of soybean oil and animal body fat was detected at a level of 5% in milk fat, while adulteration of buffalo ghee with coconut oil alone and also in conjugation with lard was detected at 5% level. Adulteration of ghee with individual body fat or soy oil a significant increase in triglycerides having 50 to 54 carbons (C50, C52 and C54) was observed and summation of these (three TGs–C50+C52+C54) markers was able to detect adulteration of ghee with body fat or vegetable oil to the tune of 5%. It was also possible to detect the adulteration of ghee with body fats (Goat tallow or lard and soy oil) if they are added in combination (Hazra, 2017). He also reported that sixteen even carbon number TGs were there in both cow and buffalo ghee, but only medium and higher carbon number triglycerides were predominant in both cow and buffalo ghee. Lower carbon number TGs were less in case of both the ghee samples. In case of goat body fat, triglycerides with 50 and 52 carbon numbers (C50

and C52 TGs) were predominant. In case of soy oil, triglycerides with 54 carbons (C54) was predominant. As a result of the admixture of goat body fat and soy oil (1:1) to both cow and buffalo ghee samples at an effective concentration of each adulterant @5% the sum of these three TGs (sum= C50+C52+C54) was increased to $47.66 \pm 1.46\%$ and 43.18 ± 2.01 , respectively.

Pathania *et al.* (2020) developed a gas chromatography-based technique for detecting RM- adjuster in cow and buffalo ghee. They observed the appearance of large sized signature peaks in the retention time range of 4.5-6.5 minutes for RM-adjuster and adulterant fat, while there was no such type of peaks in the samples of pure cow and pure buffalo ghee. Through the developed method, the addition of RM-adjusted highly adulterant fat to the minimum level 0.5% could be detected in both cow and buffalo ghee.

2.3 Reversed-phase thin layer chromatography:

Upadhyay (2014) reported that using RP-TLC the adulteration of ghee with vegetable oils can be easily detected as low as at a level of 1% on RP-18 and RP-8 plates, while RP-2 plate did not offer any help in detection of adulteration. Adulteration of ghee with coconut oil up to 7.5%, soybean oil, sunflower oil and groundnut oil up to 1%, while, designer oil up to 2% level could be detected using the standardized RP-TLC protocol (Rani *et al.*, 2015). Rakesh (2016) had reported that adulteration of ghee and butter with margarine and vegetable oils were detected at a level of 5%. He also concluded that 54.5% of ghee samples from organized sector and 66.6% of ghee samples from unorganized sector were found to be adulterated with vegetable oil. Adulteration of milk with coconut oil up to 5% level and other vegetable oils such as rice bran oil, sunflower oil, soybean oil, and groundnut oil up to 1% level could be detected using the standardized RP-TLC protocol on RP-18 and RP-8 plates (Sukthija, 2018).

2.4 Other miscellaneous methods:

2.4.1 Spectroscopic methods:

Ntakatsane *et al.* (2013) reported the great potential of fluorescence spectroscopy to rapidly discriminate between pure and adulterated milk fat. Fluorescence spectroscopy could detect up to

5% of vegetable oil in butterfat. The pure and adulterated samples were discriminated based on the total concentration of saturated fatty acids, and also on the major fluorophores: tryptophan, tocopherols, and riboflavin.

Upadhyay *et al.* (2016) reported that ATR-FTIR spectroscopy coupled with chemometrics was applied for detection of goat body fat adulteration in pure mixed ghee. They detected the adulteration of goat body fat minimum level 1% in pure mixed ghee.

Antony *et al.* (2018) used FT-MIR spectra of ghee and vegetable oils using HATR in 4000–650 cm⁻¹ region. They studied the differences in absorbance by carbon–hydrogen (C–H) stretch in fatty acid chain at 3.48 μm and absorbance by carbonyl (C=O) stretch of ester linkage at 5.7 μm in ghee and that in vegetable oils. The clear differences in the spectra of ghee and that of the vegetable oils were observed in fingerprint region, which can be very well utilized to develop FT-MIR spectroscopy as a promising tool for detection of vegetable oils adulteration in the ghee. Aparnathi *et al.* (2019) reported FT-NIR spectroscopy coupled with chemometric. They reported that the presence of foreign oil/fats in ghee at the level of 2.0% was detectable.

2.4.2 Use of electronic nose (e-nose) to detect adulteration of ghee:

Ayari *et al.* (2018) used electronic nose (olfactory machine system) to and detect the adulteration in cow ghee with different levels (10%, 20%, 30%, 40%, and 50%) of sunflower oil and cow body fat. The principal components analysis (PCA) and artificial neural networks (ANNs) methods used to analyze the generated data. Based on the results, the accuracy of the principal components analysis to detect the addition of sunflower oil and cow body fat was 96% and 97% of the data variance, respectively. The results showed, artificial neural networks identified the adulteration with sunflower oil and cow body fat with an accuracy of 91.3% and 82.5%, respectively.

2.4.3 Colorimetric methods.

Bector and Sharma (2002) reported that palm oil added to ghee can be detected at the level of 5% by turned Prussian blue color with potassium Ferricyanide & Ferric chloride reagent on the basis of tannins which are assumed to be naturally present as impurities in palm oil. To determine this test, take 2 mL of melted adulterated ghee sample and mixed with 5 mL acetonitrile in a test tube. The contents are shaken well and centrifuged. The lower layer

Acetonitrile is properly mixed with 1 mL of 0.008M ferric chloride and 0.3 mL of 0.003M potassium ferricyanide reagent solution. The Prussian blue color indicates the presence of palm oil in milk fat. However, limitation of this method was that the ghee samples/palm oil having BHA as antioxidant also gave positive test.

Sharma *et al.* (2007) have shown that minimum 7.5% level of vegetable oil in ghee can be easily detected using rapid color based test. They used 1.0 ml of a color developing reagent i.e. ($\text{H}_2\text{O} : \text{H}_2\text{SO}_4 : \text{HNO}_3 :: 20:6:14$) in a mixture of molten fat and hexane (1:1.5). The appearance of a distinct orange tinge in the upper layer indicated the presence of vegetable oils.

Joshi (2015) reported that minimum 5% level of vegetable oil in ghee can be easily detected using rapid color based test. In this test, 1 ml of ghee sample, 2 ml of DPPH (11.41×10^{-5} Mol/litre) solution and 3 ml of ethyl acetate as solvent was standardized for checking adulteration of non-cotton tract ghee (NCT) and cotton tract (CT) ghee samples with cotton seed oil. If Halphen's test is positive and if sample reduces DPPH (2 ml of 11.41×10^{-5} Mol/liter concentration) i.e., violet color disappears in less than 10 min, presence cotton seed oil as an adulterant is confirmed. If Halphen's test is positive and if sample does not reduce DPPH within 10 min, it shows absence of cotton seed oil as an adulterant and positive Halphen's test is solely due to cotton seed feeding practices.

Joshi (2015) also used ABTS assay for the detection of cotton seed oil adulteration in pure ghee and concluded that it was not able to differentiate pure ghee and cotton seed oil adulterated ghee samples.

Ramani *et al.* (2018) developed chromogenic test to detect adulteration of ghee with palm oil. They used 1.0 ml of pure ghee or adulterated ghee (5, 10, 15 and 20 percent level of adulteration of palm oil) samples and allowed to react with 2.0 ml of DPPH solution 50 mg/ 100 ml (ethanol); thereafter the color change was observed. They reported that for pure ghee the color remained violet and for adulterated ghee the color was changed from violet to yellow. The detection level of developed chromogenic test was up to 5%.

Sushma *et al.* (2018) reported a colorimetric test, wherein 5ml of molten pure ghee and market ghee samples in separate test tubes. About 5.0ml of Conc HCl was added to each of the

samples and observed for a color change after 10-15mins. For pure ghee there was no change in color while ghee adulterated with vegetable oil showed red or reddish-brown color.

Ramani *et al.* (2019) developed a rapid chromogenic method to detect adulteration of ghee which was based on the previous worker (Bector and Sahrma, 2007). In this method 2.0 ml of pure ghee or adulterated ghee (5, 10, 15 and 20 percent level of adulteration) samples was mixed with 1.0 ml of chromogenic solution i.e., 1.0 ml of Ferric Chloride solution (0.008 M) followed by the addition of 0.3 ml Potassium Ferricyanide solution (0.03 M). Contents were mixed for 30 seconds; thereafter the color change was observed. For pure ghee the color remained green and for adulterated ghee the color was changed from green to blue. The above protocol also detected 5% level palm oil adulteration in ghee.

Nimbkar and Shanmugasundaram (2019) used hunter lab (color Flex EZ) and expressed as lightness (L^*), redness (a^*) and yellowness (b^*) system. They reported that the addition of soyabean oil to ghee L^* and a^* did not show any significant deviation but b^* value decreased with increasing the level of soyabean oil adulteration. Through that instrument that could easily be detected up to 20 % of adulteration of soyabean oil in pure cow ghee.

2.4.4 Paper based assay for antioxidant detection in foods

It is evident from the above discussion especially the methods based on color development (DPPH, ABTS or Ferric chloride- Potassium Ferricyanide) that antioxidants present in refined vegetable oils were the basis of color change. As per the regulations on Ghee (FSSAR, 2011), no antioxidant or additive has been permitted in ghee. Hence, the presence of any refined vegetable oil in ghee will bring about a change in color or development of color in the presence of above said reagents (ABTS/ DPPH etc.). In the subsequent subsections, the literature related to the antioxidants and paper-based assay for antioxidant detection in foods has been reviewed to explore the possibility of developing a user friendly or convenient to use paper-based strip/ disc to detect the adulteration of ghee with refined vegetable oils.

2.4.4.1. Antioxidant

Antioxidants are a group of substances which, when present at lower concentrations, in relation to oxidizable substrates, significantly inhibit or delay oxidative processes, while often being

oxidized themselves. The applications of antioxidants have been widespread in the food industry for decades; and are in use in avoiding lipids from oxidative deterioration (Vaya & Aviram, 2001). Free radical reactions play a vital role in oxidative deterioration of lipids by the production of peroxides and their derivatives, which produce off-flavors in food products (Gordon, 2001).

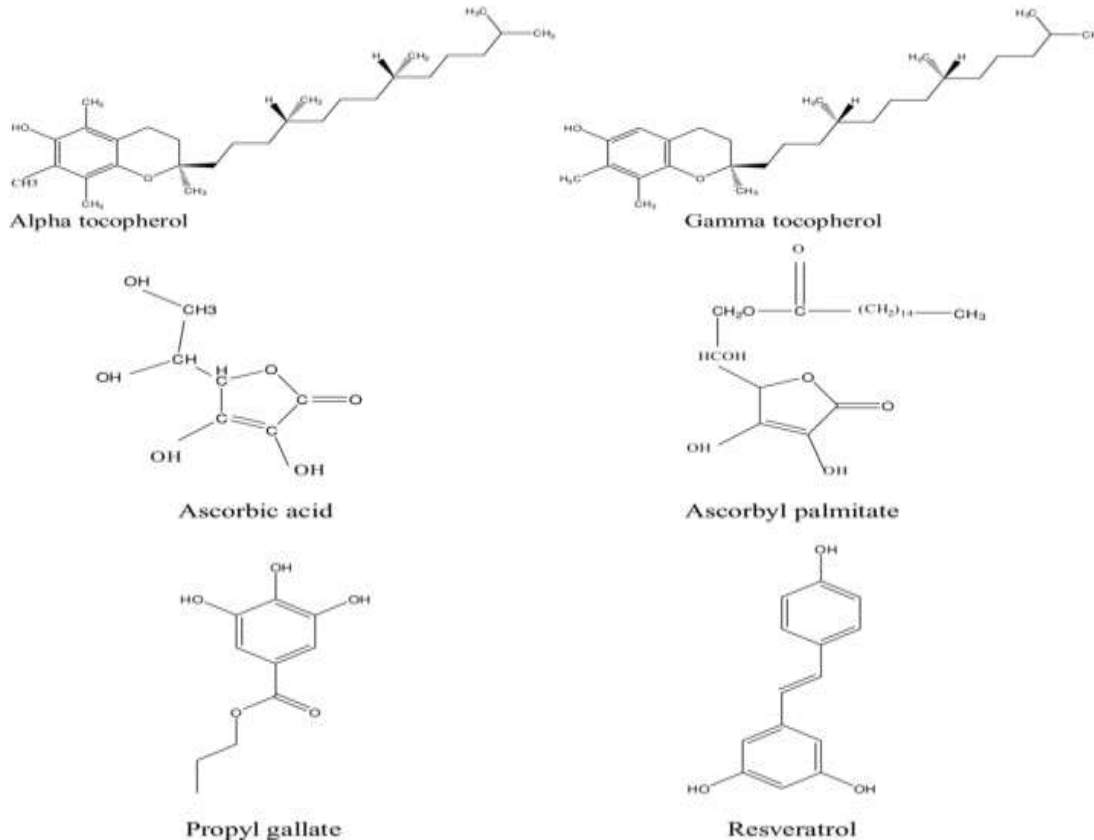
2.4.4.1.1 Classification of Antioxidants

Antioxidants are naturally existing in a wide variety of raw materials, but can also be added to food products for additional protection against oxidation. The antioxidants which are added to food products can be natural or synthetic compounds depends on their availability, and preparations (Yanishlieva-Maslarova, 2001).

2.4.4.1.1.1. Natural antioxidants

The useful effects of foods are being somewhat attributed to naturally occurring antioxidant substances (Vision et al., 1999). The search for natural sources of compounds with functional activity to be added to foods for specific population groups such as diabetics, hypertensive, etc. is important and essential. It is usually accepted, that natural antioxidant molecules are safer for human health than synthetic antioxidants, available in complex forms, which include tocopherols, lycopenes, flavonoids, nordihydroguaretic acid (NDGA), sesamol, gossypol, vitamins, provitamins and other phytochemicals, enzymes (catalase, glutathione peroxidase, super oxide dismutase), minerals (Zinc, Selenium), and lecithin (Cuppett, 2001). Alpha(α)-tocopherol (vitamin E) is well known as one of the most efficient naturally occurring lipid-soluble antioxidants McCarthy *et al.*, (2001).

2.4.4.1.1.1 Structure of Natural antioxidants



2.4.4.1.1.2. Limitations of natural antioxidants

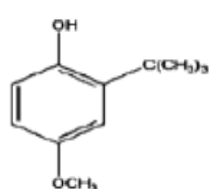
The content and composition of natural antioxidants in raw materials is variable (Cuppett, 2001), whereas, synthetic antioxidants are produced as pure substances of persistent composition. Application of synthetic antioxidants, unlike natural antioxidants, is relatively easy requiring no substantial modifications of the recipe and processing conditions. The quantity of natural antioxidant added to food products should be improved according to requirement found after analytical results such as the lesser shelf life of products due to degradation of added natural antioxidants. It is also affected by the some of the necessary processing conditions like pasteurization (reduce in vitamin E), sterilization, heating above boiling point and evaporation process which reduces some of the heat-sensitive vitamins and also decreases the biological value of proteins (Pokorny, 2001).

2.4.4.1.1.2. Synthetic antioxidants

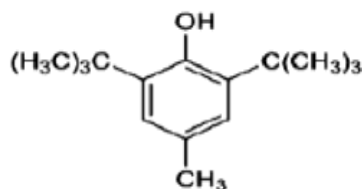
Synthetic antioxidant compounds are widely used in food products to prevent the progression of lipid oxidation. Addition of these to food products is however, banned in some countries. Its

effects in milk rely primarily on endogenous compounds (Brien & Connor, 2003). Some of the synthetic antioxidants used in so many countries including India are Butylated Hydroxy Anisole (BHA), Ter-Butyl Hydroquinone (TBHQ), and esters of gallic acid (Yanishlieva-Maslarova, 2001). Butylated Hydroxytoluene (BHT) is very effective in animal fats, low-fat food, fish products, packaging materials, paraffin and mineral oils is less effective in vegetable oils and may be lost during frying because of its steam volatility as compare to the TBHQ than BHA (Gordon & Kourimska, 1995).

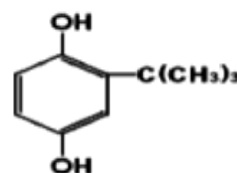
2.4.4.1.1.2.1 Structures of synthetic antioxidants



BHA



BHT



TBHQ

2.4.4.1.1.2.2 Limitations of synthetic antioxidants

There is a numerous controversy in the uses of synthetic antioxidants. Since food additives are subjected to the strictest toxicological testing procedures, only some of the synthetic antioxidants have been used in foods for any length of time. Since the toxicity of some synthetic antioxidants is not easily measured, as a result a chemical may be considered harmless by a country, tolerated in another country and forbidden in a third one. The carcinogenicity of BHA and BHT in experimental animals has been stated by Hocman (1981). Reports have revealed that BHA has carcinogenic effects in non-rodents (pigs, monkeys) and causes lesion formation in the rat stomach, whereas BHT has produced carcinogenic effects in the liver of rats and mice (Botterweck *et al.*, 2000) Thus, toxicological studies are critical in determining the safety of an antioxidant and also in determining the allowable daily intake (ADI) levels. ADIs for mostly used antioxidants such as TBHQ, BHA, BHT and gallates have altered over the years mostly because of their toxicological effects in several species. Synthetic antioxidants have limitations to be used in food requiring severe thermal treatment, as the gallates decompose when heated above 148 °C.

2.4.4.1.1.3 Antioxidant permitted in vegetable oil in India:

As per the Food safety and Standard Authority of India (2017) the permitted synthetic antioxidants for vegetable oils are as follows:

Sl. No.	Synthetic antioxidants	Permitted levels Max. (%)
1.	BHA	0.02
2.	TBHQ	0.02
3.	Ascorbale Palmitate	0.05

2.4.4.2. Cerium (IV) oxide nanoparticle

2.4.4.2.1. Materials and principle of antioxidant detection in food

Cerium(IV) oxide, also known as ceric oxide, ceric dioxide, ceria, cerium oxide or cerium dioxide, is an oxide of the rare earth metal cerium. It is a pale yellow-white powder with the chemical formula CeO_2 . It is an important commercial product and an intermediate in the purification of the element from the ores. The distinctive property of this material is its reversible conversion to a non-stoichiometric oxide. Cerium occurs naturally as a mixture with other rare-earth elements in its principal ores bastnaesite and monazite. After extraction of the metal ions into aqueous base, Ce is separated from that mixture by addition of an oxidant followed by adjustment of the pH. This step exploits the low solubility of CeO_2 and the fact that other rare-earth elements resist oxidation. (Reinhardt and Winkler, 2000).

Antioxidant detection mechanism on CeO_2 nanoparticles colloidal dispersion have been postulated with the strategy of reduction on the nano particle surface from Cerium (IV) to Cerium (III) by the antioxidants (Fig. 2.1). The reduction is followed by the color change from light yellow to dark brown of the nanoparticles which is directly proportional to the concentration and types of antioxidants.

Researchers reviewed that the antioxidant's reaction is solely correlated with the number and location of the hydroxyl groups, with *ortho* position of their function group to have increased antioxidant activity. The

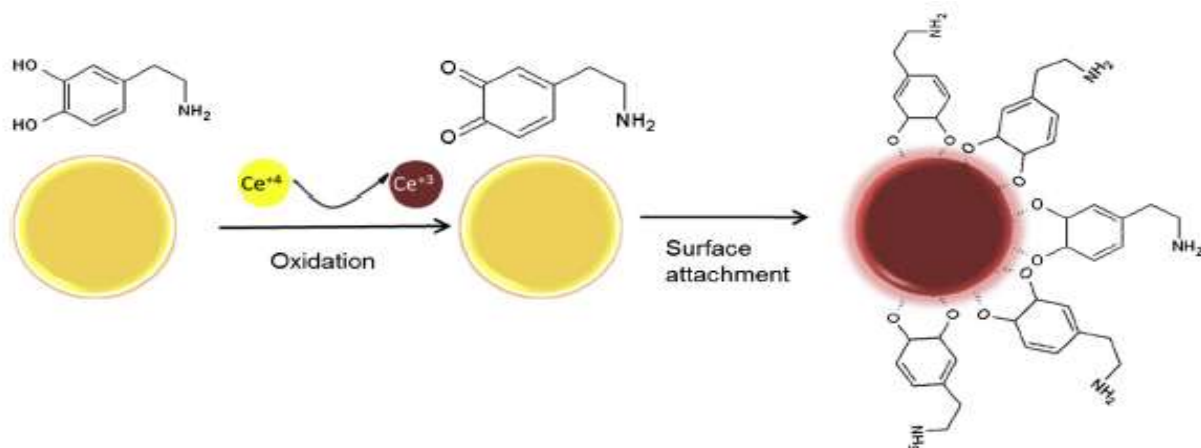


Fig 2.1: Principle of Cerium Oxide

2.4.4.2.2 Paper based CeO₂ assay for antioxidant detection in foods

Sharpe *et al.* (2012) reported the development and characterization of a portable nanoparticle based-assay for rapid and sensitive detection of food antioxidants. The assay was based on the use of immobilized ceria nanoparticles, which showed a change in color after interaction with antioxidants by means of redox and surface chemistry reactions. The sensor had been tested for the detection of common antioxidant compounds including ascorbic acid, gallic acid, vanillic acid, quercetin, caffeic acid, and epigallocatechin gallate. The developed sensor has been successfully applied for the assessment of antioxidant activity in tea and medicinal mushrooms. For antioxidant activity analysis the developed method was simple, quick response time and accurate. The detection limit was in the range between 20 - 400 mM depending on the antioxidant involved in medicinal mushroom. The developed method was validated with ORAC method by analyzing the antioxidant activity of 4 tea samples. The result showed no significance difference between both methods. The developed method was stable for over 1 year at room temperature.

Piyanan *et al.* (2018) reported a portable distance-based detection paper device that had a thermometer-like shape for rapid, instrument- free determination of antioxidant activity using a nanoceria assay. The assay was based on partial reduction of cerium ion from Ce⁴⁺ to Ce³⁺ on nanoceria deposited along the detection channel by antioxidants present in food resulting in a yellow to brown color change. The distance of the brown color on the detection channel was directly proportional to antioxidant activity, and can be easily measured using an integrated ruler without the need of any external sophisticated instrument for detection. The paper sensor was

studied for the analysis of common antioxidants and its performance was validated against traditional antioxidant assays for 11 tea sample. The antioxidant activity of tea samples obtained from the paper device correlated with the traditional assay at the 95% confidence level. The developed sensor provided a high recovery and tolerance limit and was stable for 50 days both when stored at ambient and low temperature (6 and -20°C).

Tortolini *et al.* (2018) reported a single-use electrochemical screen-printed electrode based on biomimetic properties of nanoceria particles (CeNPs). The developed sensor showed an easy approach compared to the classical spectrophotometric based methods reported in literature in terms of ease of use, cost, portability, and unnecessary secondary reagents. The sensor allowed the detection of the total antioxidant capacity (TAC) in wine samples. The sensor had been optimized and characterized electrochemically and then tested with antioxidant compounds occurred in wine samples. The electrochemical CeNPs modified sensor had been used for detection of TAC in white and red commercial wines and the data compared to the 2,20 -azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS)-based spectrophotometric method. Finally, the obtained results demonstrated that the proposed sensor was suitable for the simple and quick evaluation of TAC in beverage samples.

2.4.4.3 2, 2-diphenyl-1- picrylhydrazyl (DPPH) assay

DPPH is an organic chemical compound also called as 2,2- Diphenyl -1- picrylhydrazyl. DPPH is a dark violet colored crystalline powder composed of stable free radical molecules. It has major two applications, both in laboratory research: one is a monitor of chemical reactions involving radicals, most notably it is a common antioxidant assay and another is a standard of the position and intensity of electron paramagnetic resonance signals (Molyneux, 2004). On mixing the DPPH solution with a substance that can donate a hydrogen atom, to the DPPH gets reduced to a form with a residual pale yellow color, which is due to the picryl group still present in the structure. Representing the DPPH radical by $\text{Z}^{\bullet}$ and the donor molecule by AH , the primary reaction is where ZH is the reduced form and $\text{A}^{\bullet}$ is free radical produced in this first step.

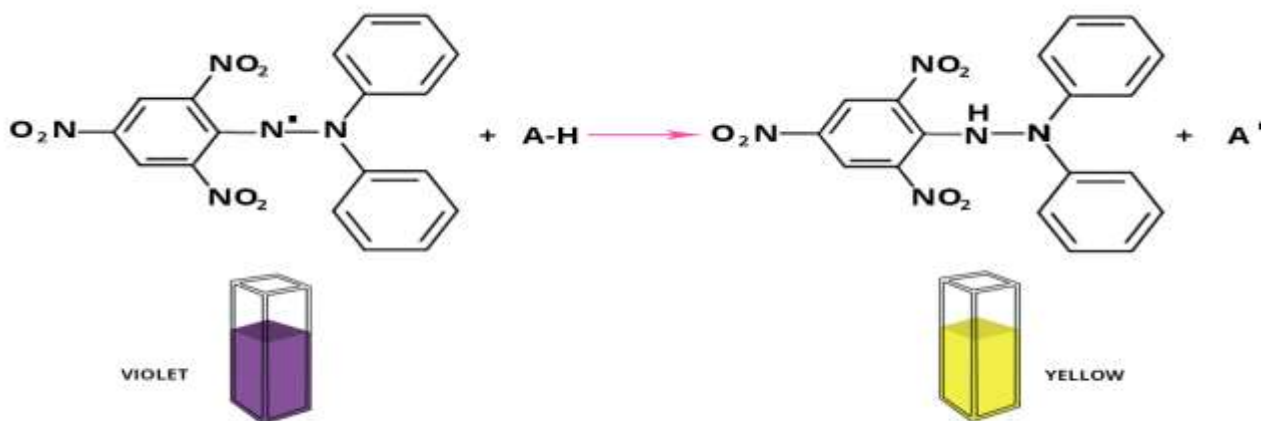
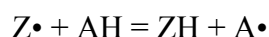


Fig 2.2: Mechanism of DPPH



This latter radical will then undergo further reactions which control the overall stoichiometry, that is, the number of molecules of DPPH reduced (decolorised) by one molecule of the reductant. The reaction is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance; the DPPH molecule $Z\bullet$ is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH (Molyneux, 2004).

The reaction of tocopherols with DPPH is very fast. Tocopherol compounds behave as effective scavengers of free radicals; the factors responsible for the high rate of reactions with DPPH are likely to be the same as those responsible for tocopherols effectiveness as an antioxidant (Rao *et al.*, 1982). This effect can be explained by its hydrogen donor capacity. Di Mambro *et al.* (2003) used DPPH to analyse antioxidant activities of tocopherols.

2.4.4.3.1 Paper based DPPH assay for antioxidant detection in foods

Musa *et al.* (2013) developed DPPH dry reagent array plate by evaporating solvent from DPPH into 96 well microplate, based on which the highly sensitive and high throughput determination of antioxidant activities was achieved. The spectrophotometric characterization of the microplate containing dried or fresh DPPH free radicals was reported. The response of the DPPH dry reagent array towards different standard antioxidants was studied. The reaction for DPPH in fresh or dry reagent array with Trolox was reported and compared. The DPPH dry reagent array was used to study the antioxidant activity of banana, green tea, pink guava, and honeydew and the results were compared to the samples reacted with freshly prepared DPPH.

The proposed method was comparable to the classical DPPH method, more convenient, simple to operate with minimal solvent required and excellent sensitivity. The stepwise procedure is given in **Fig 2.3**

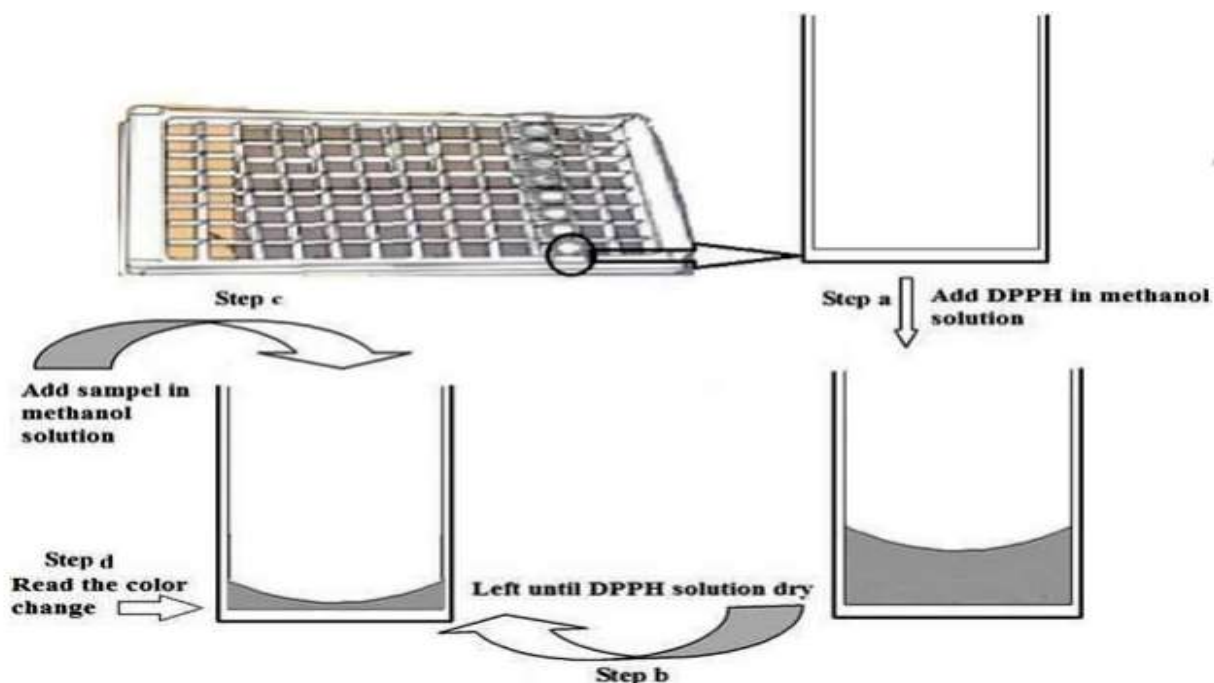


Fig 2.3 Step wise procedure for development of DPPH dry reagent array plate for detection of antioxidant in Food samples

Akar *et al.* (2017) developed a new method by using the mixtures of solutions of DPPH and standard antioxidants or extracts of common medicinal herbs were dropped onto TLC plates, after an incubation period. The spot images were evaluated with Image J software to determine CSC_{50} (Reducing the color of sample concentration at 50%) values, the sample concentrations providing 50% color reduction, which were very similar with the SC_{50} (Reducing the sample concentration at 50%) values obtained with spectrophotometric method. The developed method required lower amounts of reagents and solvents, no need for costly spectrophotometers, and thus reduces the costs, and convenient to use in any environment and situation.

Sateanchok *et al.* (2018) developed a simple cotton thread combining paper-based device with mobile phone for detection of antioxidant. Standard and sample solutions flow along with a bunch of cotton thread treated with sodium hydroxide via microfluidic behaviors without any external pumping. The analyte solution reacts with the reagents that have been immobilized on the paper strip fixed at the end of the cotton bunch. The developed platforms were used for the assays of total phenolic content and antioxidant capacity by employing Folin-Ciocalteu and 2, 2-

diphenyl-1- picrylhydrazyl (DPPH) respectively. Simple detection can be made by employing a mobile phone camera (iPhone 4S) with Image J or Photoshop for image processing and assessment. Gallic acid was used as a reference standard, as its polyphenol structures can be found in numerous plants. The total phenolic content is expressed as gallic acid equivalents (GAE) (mg/g material). IC_{50} inhibition can be assessed from the graph and can be used for the antioxidant capacity consideration. The developed method had been used to the assay of green tea samples. The total phenolic contents in the green tea samples were found to be 48 to 105 mg/g, with %RSD of less than 10 for that of higher 50 GAE mg/g and IC_{50} (inhibition concentration at 50%) values of the samples studied were 25 to 50 mg/L. The results found by the developed methods agreed with that of the standard methods. Step wise procedure for developed device for detection of antioxidant in tea samples is given in Fig 2.4

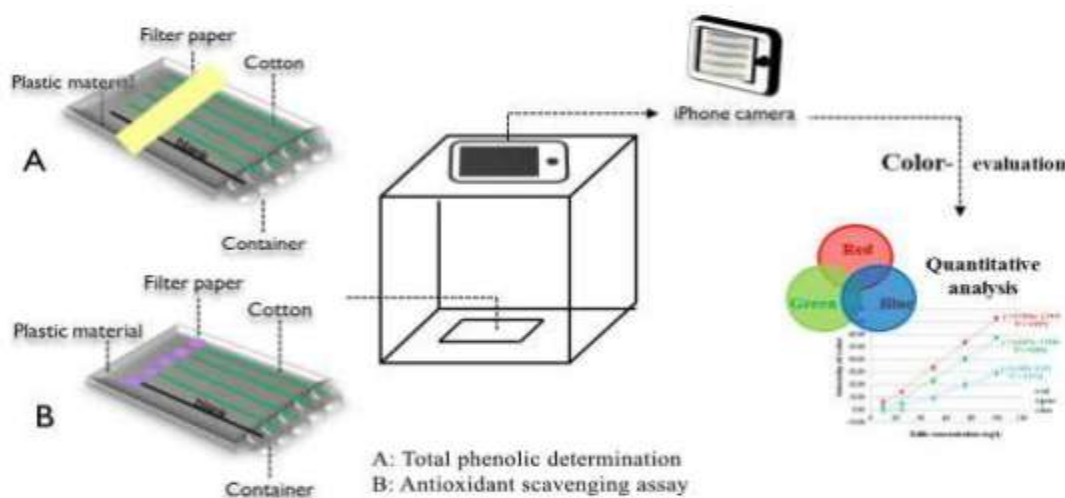


Fig 2.4 Step wise procedure for development of a simple cotton thread combining paper-based device with mobile phone detection for detection of antioxidant in tea samples

Sirivibulkovit *et al.* (2018) fabricated paper-based device for the analysis of antioxidant activity of 7 tea samples, using a lamination method to create a 5mm in diameter circular test zone that was embedded with a DPPH reagent. They carried out one step analysis by adding an antioxidant/sample onto test zone and after reduction the DPPH radicals become stable DPPH molecules, resulting in a color change from deep violet to pale yellow. The method developed had high precision and low limit of detection for the analysis of six standard antioxidant solutions including gallic acid, trolox, ascorbic acid, caffeic acid, vanillic acid and quercetin. For the validation of the developed method, traditional spectrophotometric DPPH assay was used for analyzing the antioxidant activity of 7 tea samples. The result showed no significance difference

between the two methods at the 95% confidence level, indicating the developed paper-based DPPH device, stable for over 10 days at refrigerated temperature (2-4°C), was reliable for antioxidant activity analysis of tea samples. The Step wise procedure for developed paper-based device for the analysis of antioxidant activity of 7 tea samples is given in Fig 2.5

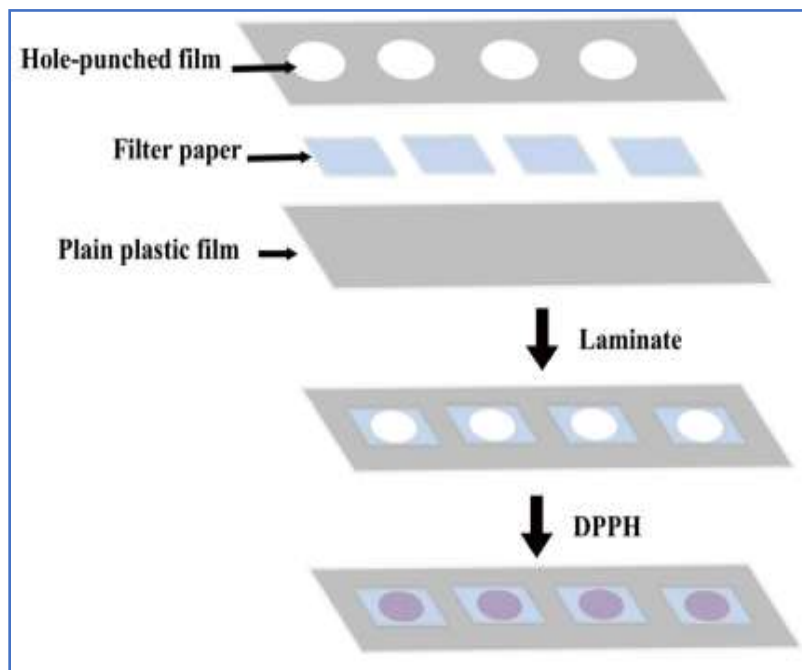


Fig 2.5 Step wise procedure for developed paper-based device

Kuswandi *et al.* (2020) developed a simple colorimetric assay of total antioxidant capacity (TAC) of the herbal extract on the paper micro zone plate (PμZP) that was constructed in the 70-well of patterned paper using the screen-printing technique given in Fig 2.6. The PμZP was constructed by immobilizing DPPH (2,2-diphenyl-1-picrylhydrazyl) through pipette onto 70-well of PμZP and dry for 10 min and sensing zone was ready for colorimetric detection. The purple-sensing zone exhibited a good response to gallic acid (GA), by producing slightly gray to pale yellow color that could be captured using a scanner and then analyzed using the ImageJ program. The paper-based sensor showed a linear response toward GA at 0.05–0.6 mM ($r=0.9895$), reproducible response ($RSD < 4\%$), and accurate measurement with 91 to 106% recovery for measuring TAC of herbal extract, presented as mM gallic acid equivalent and showed a good agreement with the standard DPPH method. The results suggested that the proposed method can be applied for simple TAC measurement in the herbal extract.

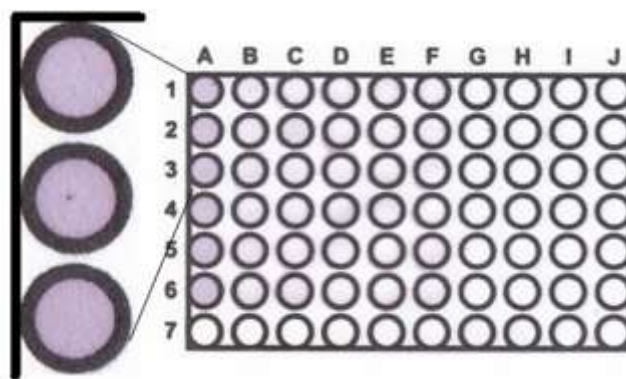


Fig 2.6 Paper micro zone plate (PuZP) that was constructed in the 70-well of patterned paper using the screen-printing technique

The comparison of commonly used methods for the detection of antioxidants in food samples are given in table 2.1 as follows:

Table 2.1 Comparison of commonly used antioxidant test in foods:

Method	Principal	Stability	Assay Conc.	Steps, Time	Assay condition	Application	Reference
Cerium Oxide	Ceria reduction	High stable 1 year	0.2-9mM	1 step, <10 min	Portable less sample dilution	Tea & Medicinal Mushroom	(Sharpe <i>et al.</i> , 2012)
DPPH	H transfer	1 month, 4°C, dark	0.3-2mM	1 step, 30 min	Light sensitive	Ghee and tea	(Sirivibulkovit <i>et al.</i> , 2018)
ORAC	H transfer	3 months	8nM-50 µM	7 steps, 1 hours	High inters assay variability	Food & Botanical Nutraceutical	(Pellegrini <i>et al.</i> , 2003)
FRAP	Electron donation	TPTZ;2-8°C, FeCl ₃ ; RT	0.1-1mM	4 steps, 2 hours	Low pH required	Tea and veg. analysis	(Benzai and Strain, 1996)
CUPRAC	Copper reduction	6 months	µM	4 steps, 2 hours	Neutral pH	Vegetable, juice & tea	(Apak <i>et al.</i> , 2004)

2.4.5 Changes during storage of ghee

In spite of its intrinsic shelf-life stabilizing properties, ghee does eventually spoil mainly due to development of oxidized flavour as a result of the oxidation of the fatty acids during storage at ambient temperature and/or rancid flavours due to lipolysis (Van den Berg, 1988).

Asha *et al.* (2015) studied the effect of storage temperature (6 ± 2 °C; 32 ± 2 °C and 60 ± 2 °C) up to 21 days on peroxide, TBA value and free fatty acid of ghee. These values were significantly higher in control ghee than ghee incorporated with orange peel extract (OPE) and BHA. Ghee added with orange peel extract (OPE) was highly stable at 6 and 32°C as compare to higher temperature 60°C stored ghee.

Divya and Vasudevan (2015) studied quality attributes of ghee based on packaging material and storage period by storing the samples in glass, PET, PP and HDPE bottles up to 60 days. They reported an increase in free fatty acid (FFA), Refractive index (RI), Peroxide value and TBA value in ghee at every 30 days interval. They also reported that during storage glass bottle gives lower oxidation values as compared to PET, PP, HDPE whereas PET packaged ghee also showed comparable good result at same storage condition.

Gosewade (2016) reported a small increase in Reichert Meissl value, Polenske value and saponification value and a small decrease in iodine value, butyro- refractometer (BR) reading at 40°C and residual BHA content during storage at 37°C. Small increase in peroxide value, conjugated diene and free fatty acid content (% oleic acid) in ghee during storage at 37°C for 8 month was also reported. It was also observed that rate of formation of peroxides and TBA reactive substances was significantly lower in ghee stored at 37 ± 1 °C and 60 ± 1 °C with added sesamol, rosemary extract (RME), γ -oryzanol as compared to control and ghee containing 0.02% BHA (Rahila, 2016). Similarly, Bhatia (2018) concluded a small rise in peroxide value, TBA value, conjugated dienes and free fatty acid content (% oleic acid) in milk fat during storage at 37°C temperature for a period of five months.

Chaudhari *et al.* (2021) observed that cow ghee and buffalo ghee adulterated separately with palm oil and subsequently stored at 80°C for 15 days, showed a small increase in Reichert Meissl value and a small decrease in butyro- refractometer (BR) reading, whereas in case of peroxide value was found much higher in all adulterated ghee samples at same storage temperature as compare to the cow and buffalo ghee samples.

CHAPTER - 3

Material and Methods

Material and methods

This chapter deals with the materials and methods used for standardization of methodology for detection of vegetable oils adulteration in ghee.

3.1 Materials:

3.1.1 Chemicals:

2,2-diphenyl-1-picrylhydrazyl [DPPH; (C₁₈H₁₂N₅O₆)] and Cerium (IV) oxide (CeO₂) nanoparticles/ceria i.e. 20% colloidal dispersion in 2.5% acetic acid(CH₃COOH) were purchased from Merck Specialities Pvt Ltd., Mumbai, India; Trichloroacetic acid(C₂HCl₃O₂) was purchased from Sisco Research Laboratory Pvt. Ltd, Maharashtra, India; Hydrochloric acid (HCl) was purchased from Thermo Fisher Scientific, India Pvt. Ltd; TBA : Thio Barbituric acid (C₄H₄N₂O₂S), Anhydrous sodium sulphate (Na₂SO₄), Glacial Acetic acid (CH₃COOH), Iodate free Potassium iodide (KI), Potassium iodate (KIO₃), Sodium Carbonate (Na₂CO₃), sodium thiosulphate(Na₂S₂O₃), AR grade; Sodium sulphate anhydrous (Na₂SO₄) and Phosphomolybdic acid (H₃PMo₁₂O₄₀) were purchased from Titan Biotech Ltd Bhiwadi, India.

3.1.2 Solvents:

Methanol (HPLC grade) was purchased from Thermo Fisher Scientific India Pvt Ltd; n-Hexane was purchased from Honeywell International India Pvt. Ltd.; Chloroform (AR grade), Ethanol absolute and Acetonitrile (HPLC grade) were purchased from S.D Fine- chem. Ltd. Mumbai, India; Petroleum Ether (AR grade) purchased from Glaxo Laboratories India Ltd., Bombay; Ethyl Acetate and Diethyl Ether were purchased from Merck Specialities Pvt Ltd., Mumbai

3.1.3 Synthetic antioxidants:

Butylated Hydroxyanisole (BHA), Butylhydroquinone (TBHQ) and Ascorbic acid were purchased from Sigma Aldrich, St. Louis, USA; Butylated Hydroxytoluene (BHT) was procured from Thermo Fisher Scientific India Pvt Ltd; Gallic Acid was procured from central Drug House (P) Ltd, New Delhi, India: 6-O-Palmitoyl-L-ascorbic Acid was procured from Tokyo Industry CO., LTD 6-15-9 Toshima, Kita-Ku, Tokyo, Japan;

3.1.4 Reversed - phase thin layer chromatography (RP- TLC) plate:

TLC Aluminium sheet Silica Gel 60 RP-18 F₂₅₄S TLC plates were purchased from Merk Specialities Pvt. Ltd. Mumbai.

3.1.5 Filter papers:

Whatman filter paper no-1 and 4 and thick Chromatographic filter paper (3 mm CHR) were purchased from Whatman International Ltd., Kent, England.

3.1.6 Reagents:

3.1.6.1 Stock solution (0.02%) of BHA and TBHQ:

Dissolved 20 mg of synthetic antioxidant (BHA & TBHQ) separately in acetonitrile and volume was made to 100 ml in volumetric flask, as stock solutions.

3.1.6.1.1 Working solution of BHA and TBHQ:

The above-mentioned stock solution was used to prepare the working solutions of lower concentrations (0.01, 0.005 and 0.0025%) in acetonitrile.

3.1.6.2 Stock solution (0.05%) of ascorbyl palmitate (6-O-Palmitoyl-L-ascorbic Acid):

Dissolved 50 mg of **6-O-Palmitoyl-L-ascorbic Acid** in small quantity of ethanol and volume was made up to 100 ml in volumetric flask, as stock solution.

3.1.6.2.1 Working solution of 6-O-Palmitoyl-L-ascorbic Acid (AsP):

The said stock solution was used to prepare the working solutions of lower concentrations (0.02, 0.01, 0.005 and 0.0025%) in ethanol.

3.1.6.3 Stock solution (10 mM) of 6-O-Palmitoyl-L-ascorbic Acid (AsP):

Dissolved 41.45 mg of **6-O-Palmitoyl-L-ascorbic Acid** with ethanol and methanol, separately and made up the volume to 10 ml with respective solvents, as a stock solution.

3.1.6.3.1 Working solution (10 mM) of 6-O-Palmitoyl-L-ascorbic Acid (AsP)

The above said stock solution was used to prepare the working solutions of lower concentrations (8, 6, 4, 2 and 1) mM in their respective solvents, separately.

3.1.6.4 Preparation of Ascorbic Acid (10 mM) solution for standard antioxidant (stock solution):

Dissolved 17.612 mg of **Ascorbic Acid** in a 10 ml volumetric flask with ethanol as a stock solution, as stock solution.

3.1.6.4.1 Working solution of Ascorbic Acid:

The above said stock solution was used to prepare the working solutions of lower concentrations (8, 6, 4, 2 and 1) mM were prepared in ethanol.

3.1.6.5 Preparation of Ascorbic Acid (0.03%) solution for standard antioxidant (stock solution):

Dissolved 30 mg of ascorbic acid in ethanol and volume was made up to 100 ml in volumetric flask, as stock solution.

3.1.6.5.1 Working solution of Ascorbic Acid:

The above said stock solution was used to prepare the working solutions of lower concentrations (0.02, 0.01, 0.005 and 0.0025) % were prepared in ethanol.

3.1.6.6 Preparation of Gallic Acid (2 mM) solution for standard antioxidant (stock solution):

Dissolve 3.4 mg of **Gallic Acid** in a 10 ml volumetric flask with acetonitrile, as a stock solution.

3.1.6.6.1 Working solution of Gallic Acid

The above said stock solution was used to prepare the working solutions of lower concentrations (1.5, 1 and 0.5) mM were prepared in acetonitrile

3.1.6.7 Gallic Acid (0.02 %) solution for standard antioxidant (stock solution):

Dissolve 20 mg of **Gallic Acid** in a 100 ml volumetric flask with acetonitrile as a stock solution.

3.1.6.7.1 Working solution Gallic Acid

Further, the working solutions of lower (0.01, 0.05 and 0.0025) % were prepared by diluting the stock solution with acetonitrile in each concentration, separately.

3.1.6.8 Hydrochloric acid (6.0M) solution

Took 5.0 ml of concentrated hydrochloric acid and the content were diluted to 10 ml with distilled water.

3.1.6.9 0.1N Sodium Thiosulphate (Na₂S₂O₃) solution (stock solution)

Dissolved 25 g of Na₂S₂O₃ crystals in freshly boiled and cooled (to make it free from CO₂) distilled water and volume was made up to 1000 ml. The solution was stored in a dark-colored reagent bottle and standardized as follows:

About 0.10 - 0.15 g of the potassium iodate as a primary standard was accurately weighed and transferred to a clean Erlenmeyer flask (250 ml). Dissolved the content in 75 ml of distilled water and add 2.0 gm of iodate free potassium iodide. Then add 2.0 ml of 6.0 M HCL and titrate immediately with 0.1 N Sodium Thiosulphate (Na₂S₂O₃) solution until the solution become yellow, shaking constantly and vigorously during titration. Add 0.5ml of starch indicator solution. Blue /violet color will appear. Continue titrate with (0.1N) sodium thiosulphate dropwise until the blue /violet color disappears. Record the total volume of added sodium thiosulphate solution. Repeat the titration twice/thrice to record the exact volume of (0.1N) sodium thiosulphate used. Carry out the blank titration in a similar manner, without taking the primary standard. The normality of Na₂S₂O₃ solution was calculated as follows:

$$\text{Normality of Na}_2\text{S}_2\text{O}_3 \text{ solution} = \frac{28.037 \times W}{S \times B}$$

Where,

W = weight of KIO₃ taken in gm

S = volume of Na₂S₂O₃ required for titration of sample in ml

B = volume of Na₂S₂O₃ required for titration of blank in ml

3.1.6.10 Sodium Thiosulphate (Na₂S₂O₃) solution (0.002 N):

Prepared freshly by diluting the previously prepared 0.1 N Sodium Thiosulphate (Na₂S₂O₃) stock solution.

3.1.6.11 Saturated Potassium iodide solution:

Added enough potassium iodide to freshly boiled and cooled distilled water to ensure a saturated solution (10.0 gm KI in 6.0 ml water). Stored it overnight to ensure that un-dissolved crystals

remained there. To test stability, diluted 0.5 ml saturated KI solution in 30 ml of 3:2 (V/V) acetic acid/chloroform solution and then added 2 drops of 1% starch indicator solution.

3.1.6.12 Starch indicator solution (1% w/v):

One gram of soluble starch was weighed and paste was made in 30 ml of water. The paste was transferred to 80 ml boiling water and heated until a clear solution was obtained. The contents were cooled and stored in a tightly stoppered bottle.

3.1.6.13 Mixed Solvent for peroxide value:

Mixed solvent was prepared by mixing glacial acetic acid and chloroform in 2:1(V/V).

3.1.6.14 Trichloro acetic acid (35% w/v):

Dissolved 35 g of trichloroacetic acid in distilled water and volume was made up to 100 ml in a volumetric flask.

3.1.6.15 Thio Barbituric acid (TBA) solution:

0.36g of TBA (2- Thiobarbituric acid) and 0.1 g of anhydrous sodium sulphate were weighed accurately and dissolved in distilled water. The volume was made up to 100 mL using volumetric flask.

3.1.6.16 Solvent system for RP-TLC:

Petroleum ether, Acetonitrile and Methanol were mixed in 2:4:4 v/v proportion.

3.1.6.17 Phosphomolybdic acid solution (20% solution in ethanol)

20g of Phosphomolybdic acid was weighed accurately and dissolved in ethanol. The volume was made up to 100 mL using volumetric flask. Further it was filtered through what men filter paper no-1 and then kept it in refrigerated temperature for further use.

3.1.7 Apparatus and glassware:

Burette (50 ml), funnels (75mm diameter), measuring cylinder (5, 10, 50, 100 ml), beakers (50, 100, 150, 250 and 500 ml), conical flasks (25, 50, 150, 250, 500 ml), volumetric flasks (5, 10, 25, 50, 100 and 250ml), glass pipettes (5 and 10 ml), flat bottom flasks (250 ml) were purchased

from Borosil India Ltd., Mumbai, India. Glass cuvettes (4 ml) were procured from Axiva Industries Pvt. Ltd, Ambala, India, Automatic pipettes (1 to 20 µl, 20 to 200 µl and 100 to 1000 µl) were procured from Thermo Fisher Scientific, India; HDPE narrow mouth clear and amber colored bottle (30 ml), microcentrifuge clear (1.5 and 2ml) and amber color (2 ml) were procured from Tarson products Pvt. Ltd. KolKata.

3.1.8 Equipment:

3.1.8.1 Gas Chromatography: GC-2010 Plus, Shimadzu, Japan

3.1.8.2 Boiling Water Bath: The Laboratory Glassware Co., Ambala Cantt., India

3.1.8.3 Hot Air Oven: Tempo industrial corporation, Bombay, India

3.1.8.4 Electric Heater: Vikrant, Jain Enterprises, India

3.1.8.5 Magnetic stirrer: Digital, Spinot, Tarsons Products Pvt Ltd., India

3.1.8.6 Centrifuge (REMI): Remi udyog, Bombay-2., India

3.1.8.7 UV- VIS single beam Spectrophotometer: WSP-UV-550, 220V, WENSAR WEIGHING SCALES LIMITED, New Delhi, India.

3.1.8.8 Refrigerator: LG -electronics, India Pvt. Ltd.

3.1.8.9 Vortex mixer: Spinix, Tarsons Product Pvt. Ltd., India

3.1.8.10 Weighing Balance: Precisa 310 M, Adair, Dutt & Co (I) Pvt Ltd., Calcutta

3.1.9 Procurement of pure cow ghee and adulterant vegetable oils

3.1.9.1 Collection of pure cow ghee

Pure cow ghee was collected from the Experimental Dairy (ICAR- NDRI, Karnal, Haryana).

3.1.9.2 Procurement of adulterant vegetable oils

The refined vegetable oils namely sunflower oil and palm oil used in the study were purchased from the local market at Karnal, Haryana.

3.1.9.3 Procurement of market ghee samples

Total six branded and unbranded ghee samples were purchased from local market of Karnal, Haryana. The samples are mentioned in code as follows (Table 3.1).

Table 3.1 List of branded and unbranded ghee samples with code used in the study

SL. No.	Branded Ghee samples Code used in the study	Unbranded Ghee samples Code used in the study
1	A	1
2	B	2
3	C	3
4	D	4
5	E	5
6	F	6

3.2 Methods:

3.2.1 Optimization of DPPH concentration for efficient visual color change in presence of antioxidants (BHA, TBHQ, Gallic acid, Ascorbic acid and 6-O-Palmitoyl-L-ascorbic Acid)

DPPH solution of (1, 2, 3 and 4) mM concentration was prepared in methanol. Then, from each DPPH solution (1, 5, 10, 15, 20 & 25) μL were pipetted out and transferred to 600 μL of standard antioxidant BHA, TBHQ and 6-O-Palmitoyl-L-ascorbic Acid (AsP) solution (0.0025, 0.005, 0.01 & 0.02%) solution, respectively.

Gallic acid (0.02%) and Ascorbic acid solutions (0.03%), which was permitted as per FSSAI regulations. The contents were mixed and kept at room temperature to observe time elapsed to change color from violet to yellow or disappearance of violet color. Based on the visual observation of DPPH color and significant different in timing to change color from all concentrations of BHA, TBHQ and 6-O-Palmitoyl-L-ascorbic Acid the final concentration was selected.

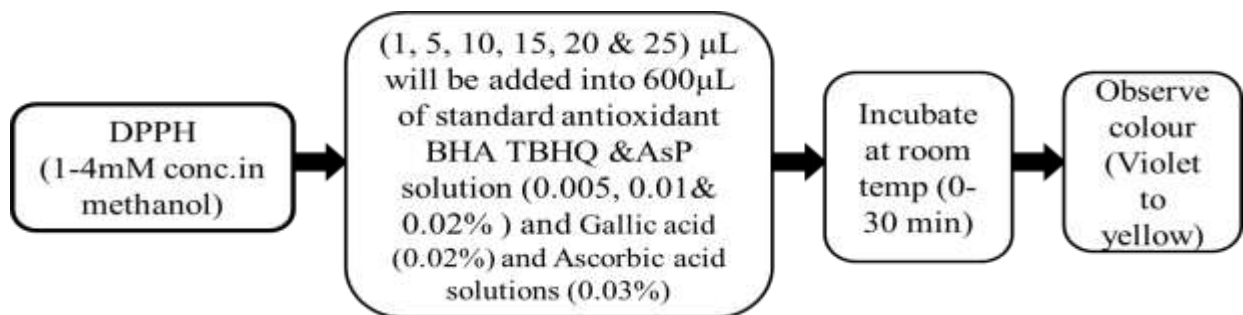


Fig. 3.1 Flow chart for optimization of DPPH concentration for efficient visual color change in presence of antioxidants (BHA, TBHQ, GA, AA and AsP)

3.2.2 Optimization of Ce (IV) O₂ dispersion concentration for efficient visual color change in presence of antioxidants (BHA, TBHQ, Gallic acid, ascorbic acid and 6-O-Palmitoyl-L-ascorbic Acid)

Cerium (IV) oxide (CeO₂) nanoparticles/ceria i.e., 20% colloidal dispersion in 2.5% acetic acid was taken and from that, varied quantity (1, 5, 10, 15, 20 & 25 μl) was taken and added into 600 μl of standard antioxidant i.e BHA and TBHQ and 6-O-Palmitoyl-L-ascorbic Acid solution which were prepared in different concentration (0.0025, 0.005, 0.01 & 0.02%). Similarly, in case of Gallic acid (0.02%) and Ascorbic acid (0.03) % solution, 600μL of each was taken and to those different volumes of Cerium (IV) oxide were added. After that, it was kept at room temperature for observation of timing to change color from yellow to brown or disappearance of yellow color. Based on the visual observation of CeO₂ color change from all concentrations of BHA, TBHQ and 6-O-Palmitoyl-L-ascorbic Acid the final concentration was selected.

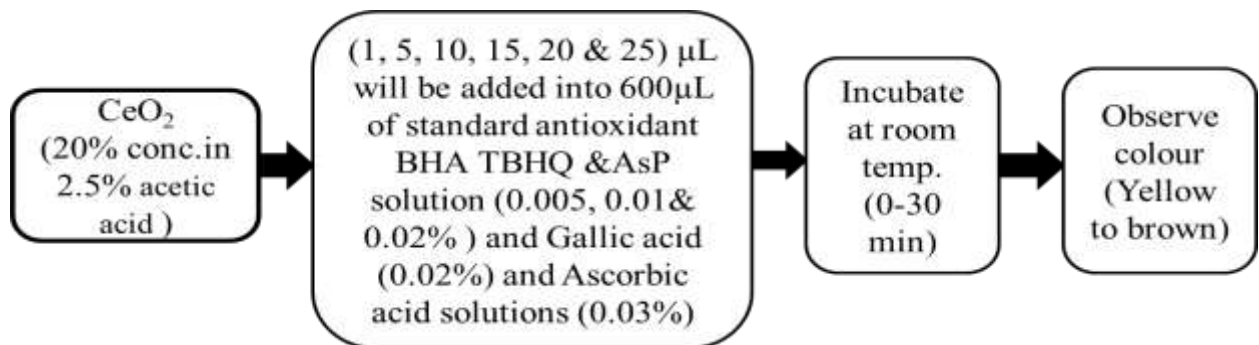


Fig. 3.2 Flow chart for optimization of Ce (IV) O₂ dispersion concentration for efficient visual color change in presence of antioxidants (BHA, TBHQ, GA, AA and AsP)

3.2.3 Optimization of conditions for the efficient extraction of antioxidants from ghee samples

3.2.3.1 Preparation and storage of adulterated ghee samples

Cow ghee was heated at 45°C and mixed well to ensure the homogeneity in the sample. Stock solution of ghee with 5% antioxidant (BHA & TBHQ) was prepared in the above said pure cow ghee. Ghee samples with effective concentration of antioxidants @ 0.0025, 0.005, 0.01 & 0.02% antioxidant were prepared by diluting the stock ghee solution, with pure ghee devoid of any added oxidant.

In another set, the adulterant vegetable oils (Palm oil and sunflower oil) were added individually to pure cow ghee @ 1, 2.5, 5, 7.5 & 10 % to obtain the ghee samples spiked with different adulterant vegetable oils.

After the addition of synthetic antioxidants and adulterant oils to ghee, the samples were thoroughly mixed, filled stored in air tight HDPE narrow mouth bottles and stored in the room temperature (30°C) till further analysis.

3.2.3.2 Standardization of protocol for efficient extraction of antioxidants from ghee samples

In this experiment attempt was made to evaluate the effect of solvents and single or double extraction on the extraction of antioxidants from ghee. Hence, the following set of experiments were carried out:

One milliliter of antioxidant spiked ghee sample was mixed with 1.0 ml of hexane followed by mixing and addition of 1.0 ml of acetonitrile, in another set of experiment 1.0 ml of antioxidant spiked ghee sample was mixed with 1.0 ml of hexane and then 2.0 ml solvent (hexane and acetonitrile mixture in the ratio of 1:1 & 1:2) in 15 ml of centrifuge tube. The above mixture was vortexed for 30 seconds for a single extraction. In one condition, it was centrifuged at 2000rpm for 5 min and, in another condition, it was kept undisturbed for 5-10 minutes. Separately, the solvent layer was collected in 2ml micro-centrifuge tubes in both of the above-mentioned conditions. Based on the upper layer clarity/without air bubbles, only one condition was selected. After that, the double extraction repeated the same procedure by adding only 1 ml

of acetonitrile to a selected condition. Again, the clear upper layer from the double extraction was collected in a 2 ml micro-centrifuge tube. The extraction of antioxidants from the sample is given in table 3.2:

Table 3.2 Single and double extraction procedure of antioxidants from ghee samples

Extraction	Pure ghee	Hexane (ml)	Acetonitrile (ml)	Upper Layer Volume (ml)	Total Volume Extracted(ml)
Single	1	1	1	600 μ L	600 μ L
Double	1	1	1	600 μ L	About
	-	-	1	About 1400 μ L	2000 μ L

The optimized concentration of DPPH was added to 600 μ L portion of extracted antioxidant in each tube.

3.2.4 Fabrication of Card, disc and tube Sensor

3.2.4.1 Fabrication of card sensor

The card sensor was fabricated by using a lamination method given by Sirivibulkovit *et al.* (2018). This sensor consisted of three layers, the top most layer was a plastic lamination film that was punched to have circular holes, the middle layer was of either Whatman No. 4 filter paper or chromatographic paper and the bottom layer was plain plastic lamination film. The three layers were heat laminated to produce a card sensor with a circular detection zone. The optimized concentration 4 mM and 20 μ L of DPPH and CeO₂ was added into the disc in (1, 5, 10, 15, 20 and 25 μ L) and evaporated at ambient temperature (30°C).

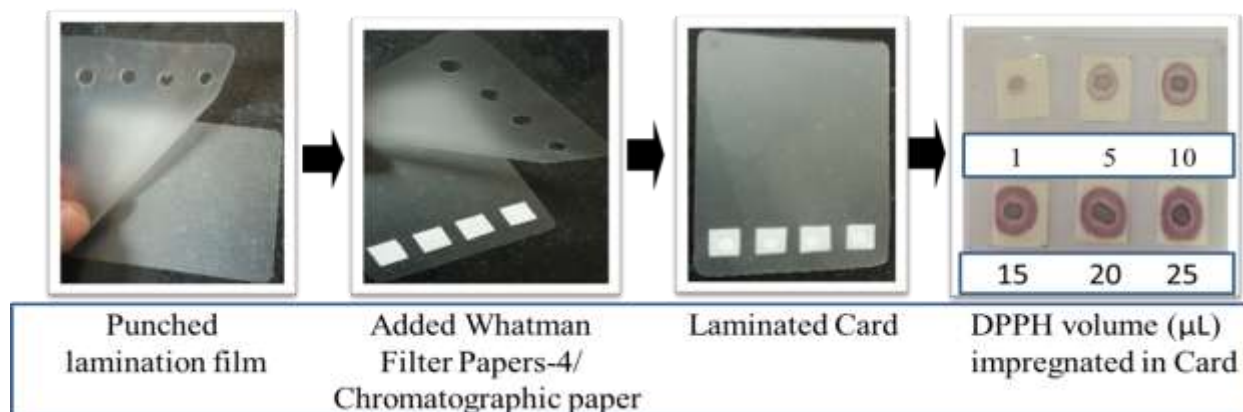


Fig. 3.3: Flow chart for fabrication of card sensor

3.2.4.2 Fabrication of disc sensor

Disc sensor was fabricated according to the method given by Sharpe *et al.* (2012). In this set of experiments, two types of filter papers i.e., whatman filter paper 4 and chromatographic paper were evaluated. These papers were cut into small circular discs (0.5 cm diameter) and small squares of approximately (0.5x0.5 cm) by using a regular paper punching machine and seisor, respectively. The optimized concentration 4 mM and 20 μL of DPPH and CeO_2 20% dispersion was added into the disc in (1, 5, 10, 15, 20 and 25 μL) and evaporated at ambient temperature (30°C) in case of DPPH and for CeO_2 (85°C) for 5 min. (Fig 3.2 and 3.3)

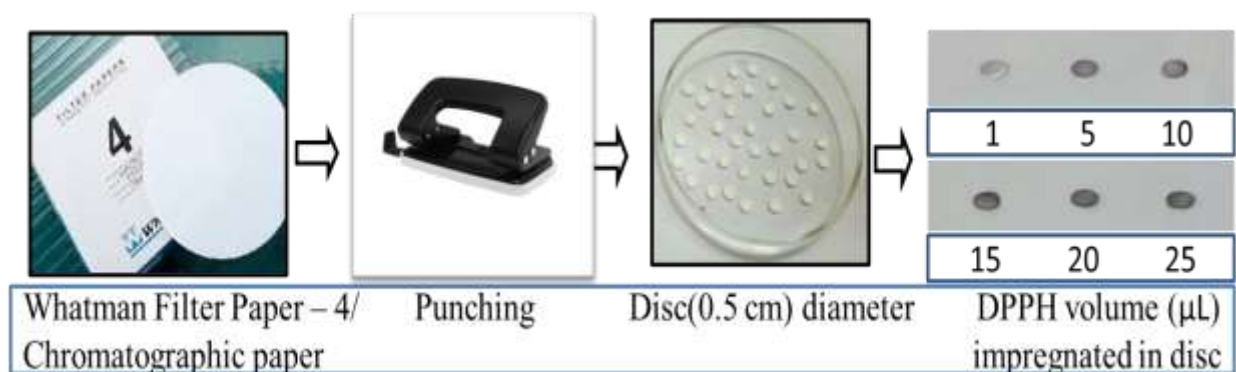


Fig. 3.4: Flow chart for fabrication of circular disc sensor

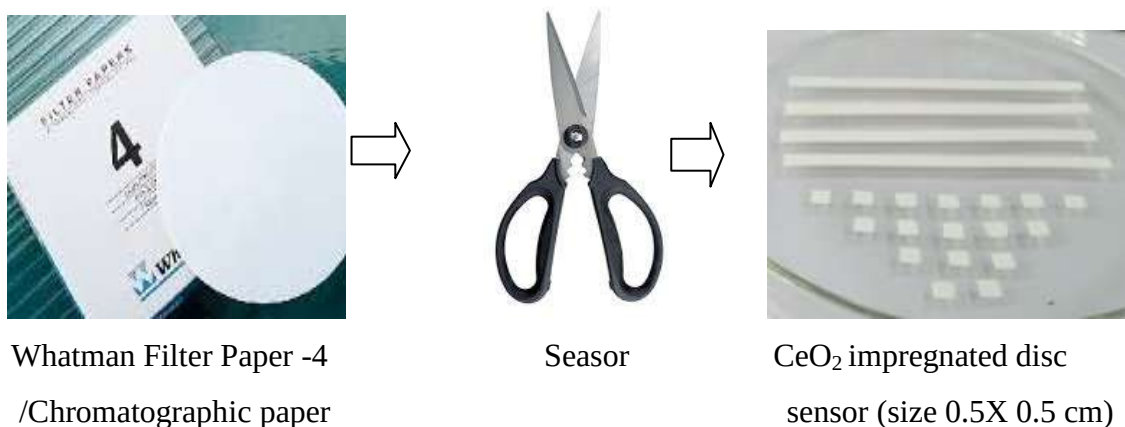


Fig. 3.5: Flow chart for fabrication of square disc sensor

3.2.4.3. Fabrication of tube sensor

The tube sensor was fabricated by using a developed DPPH dry reagent tube by evaporating solvent from DPPH into eppendorf tube method given by Musa *et al.* (2013). The optimized concentration 4 mM and 20 μL of DPPH was added into the 2ml of eppendorf tubes and evaporated at ambient temperature (30°C).

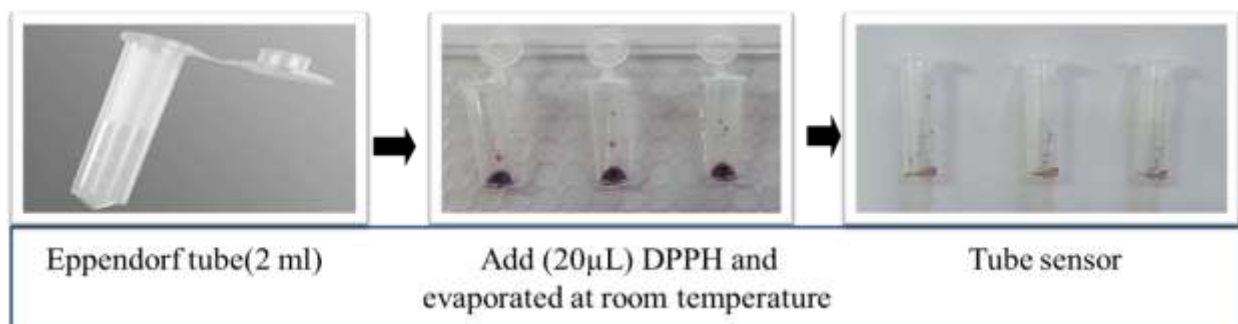


Fig. 3.6: Flow chart for fabrication of tube sensor

3.2.5 Optimization of the volume of DPPH concentration on card, disc sensor

For optimization of the above card and disc sensor's lower concentration of synthetic antioxidants BHA TBHQ and AsP (0.0025%) was used. On the basis of visible color change through naked eye from violet to yellow for DPPH and light yellow to brown color for CeO₂ the impregnated volume of dyes in above sensors was selected.

3.2.6 Storage stability of developed rapid color based sensors

3.2.6.1 Storage stability of developed rapid color-based sensor (Tube/Disc)

To evaluate the storage stability of developed rapid color-based sensors, the DPPH impregnated Eppendorf tubes (tube sensor) were packed in aluminum foil and then put inside the cardboard box. Packed sensors were then stored at refrigerated (4-6°C) and ambient temperature (30°C) for six months and evaluated periodically to check their efficiency and thereby shelf life.

The prepared Disc sensors i.e., DPPH impregnated discs were packed in HDPE amber colored bottle and HDPE clear plastic bottles. Both the bottles containing disc sensors were then stored at refrigerated (4-6°C) and ambient temperature (30°C) up to six months. These were evaluated periodically to check their efficiency and thereby shelf life.

Theses stored sensors were evaluated at 15 days interval up to 6 months. During the experiment, 600 μL of freshly prepared standard antioxidant BHA, TBHQ (0.0025, 0.005, 0.01 & 0.02%) solutions were used to evaluate the efficiency of these sensors by observing the time elapsed to change the violet color to color less or yellow color.

The end point of the non- suitability of the developed sensor was the no color change (original color of the sensor i.e. violet in DPPH based sensors) or prolonged time to change the

color after treating with standard antioxidant solutions of above-mentioned concentrations. Based on the higher storage stability any one of the sensors was selected.

3.2.6.2 Standardization of GLC conditions to evaluate the degradation of added antioxidants in stored ghee samples

The following two methods (i) specified for triglyceride analysis (ISO: 17678:2010), wherein CP-7532 (5m x 0.53mm x 0.17 μ m) column was used and (ii) Method of Yang *et al.*, (2002) for estimation of synthetic antioxidants (BHA, BHT and TBHQ) in vegetable oils., wherein SPB-5(30.0 m x 0.53 mm x 1.5 μ m) column was used.

For the determination of detection limit of both the columns, the antioxidant (BHA, BHT and TBHQ) solutions prepared in ghee and acetonitrile @ (0.0025, 0.005, 0.01 and 0.02) %, separately were used. The details are as:

In first approach (ISO: 17678:2010), to determine of detection limit of BHA and TBHQ the following protocol was adopted:

- a. 50 μ L of ghee spiked with BHA, BHT and TBHQ at 0.02% were taken separately in 5 ml volumetric flask and volume was made to 5.0 ml with hexane. Then from the above 1.5 ml transferred to vial and run on the GLC as per programme (Table 3.3)
- b. BHA, BHT and TBHQ solution prepared in ACN were directly transferred into 1.5 ml vial and run the GLC programme as per condition (Table 3.3)
- c. Extracted antioxidants from ghee spiked with BHA, BHT and TBHQ as per section 3.2.3.2. Then from the above 1.5 ml transferred to vial and run the GLC programme as per condition (Table 3.3)

In the second approach the protocol used in an earlier study in vegetable oils (Yang *et al.*, 2002) was adopted:

- a. 0.06g of antioxidants BHA, BHT and TBHQ spiked ghee sample were dissolved separately with 1 ml of diethyl ether. Then, from the above directly transferred 1.5 ml into vial and run the GLC programme as per condition (Table 3.4).
- b. BHA, BHT and TBHQ concentrations prepared in ACN were directly transferred into 1.5 ml vial and run the GLC programme as per condition (Table 3.4)

- c. Extracted antioxidants from ghee spiked with BHA, BHT and TBHQ as per section 3.2.3.2 Then from the above 1.5 ml transferred to vial and run the GLC programme as per condition (Table 3.4)

From the above two methods through all those approaches determined linearity at lower level of antioxidants. Based on the minimum level of linearity only one column was selected. The selected column was used for the degradation of BHA and TBHQ in stored adulterated ghee samples.

3.2.6.2.1 Chromatographic analysis

The following two GLC conditions (Table 3.3 and 3.3) were evaluated to resolve the pure standard antioxidants on two different columns:

Table 3.3 Conditions used for synthetic antioxidant detection in adulterated ghee samples

	Conditions
Carrier Gas	Nitrogen
Column	CP-Ultimetel SimDist CP7532 (Agilent Technologies), capillary column of 5.0 m (L) 9 0.53 mm (ID) 9 0.80 mm (OD), film thickness 0.17 lm
Column flow	3.00 mL/min of nitrogen carrier gas
Detector	Flame Ionization detector (370°C)
Injector	Split (370°C)
Column oven temperature	Initial 80°C and then increased to 190°C at the rate of 50°C/min and then to 350°C@6 C/ min and held at the final temperature for 7.0 min.
Injection vol.	0.5 µL

Table 3.4 Conditions used for synthetic antioxidant detection in adulterated ghee samples

	Conditions
Carrier Gas	Nitrogen
Column	SPB-5 (Supelco), capillary column of 30.0 m (L), 0.53 mm (ID) and film thickness 1.5 µm
Column flow	4.00 mL/min of nitrogen carrier gas
Detector	Flame Ionization detector (290°C)
Injector	Split (250°C)
Column oven temperature	Initial 150°C/1 minutes and then increased to 200°C at the rate of 7°C/min and then to 300°C@40°C/ min and held at the final temperature for 5.0 min.
Injection vol.	0.2 µL

3.2.6.1.1 Integration and evaluation of peaks:

Integration of the antioxidants present in the test sample was done using the retention time of each antioxidant present in the synthetic antioxidant standard *i.e.* BHA and TBHQ.

The area % of each antioxidant in the test sample was converted through standard curve equation in percentage concentration of antioxidants in the samples.

3.2.7 Evaluation of developed rapid color based disc sensor in stored and market ghee samples

For evaluation of disc sensors were correlated with the status of residual antioxidants in stored ghee samples as (section 3.2.3.1). The details are as in section 3.2.7.1 and 3.2.7.2.

3.2.7.1. Evaluation of oxidation in stored adulterated ghee samples:

Control and ghee samples spiked with antioxidants (BHA & TBHQ) and vegetable oils (Palm oil and Sunflower oil) were stored in HDPE plastic bottles at 30°C. The above samples were evaluated for their status of oxidation by (i) Peroxide value and (ii) TBA value at every 15 days interval up to 9 months. The methods are as follows:

3.2.7.1.1 Determination of Peroxide value of ghee:

The peroxide value (PV) of ghee was determined by iodometric method, as described in IS: 3508:1966; reaffirmed in 2018).

3.2.7.1.2. Determination of Thio Barbituric acid (TBA) value of ghee:

TBA value of ghee samples was determined by the method, as described in Asha *et al.* (2015).

3.2.7.2 Evaluation of developed rapid color based disc sensors in stored adulterated ghee samples.

Antioxidant was extracted from the stored samples as per the standardized protocol (section 3.2.3.2). The disc sensor was evaluated for its sensitivity in the extracted antioxidants.

3.2.7.3. Measurement of antioxidants level in stored ghee samples by using standardized GLC method.

The standardized method (section **3.2.6.2**) was used for the measurement of residual antioxidants levels in stored adulterated ghee samples. Antioxidant from the above samples were extracted with solvents as per the standardized protocol mentioned in section **3.2.3.1**. from the extracted solvent 1.5 ml transferred into 1.5 ml vial. Directly injected 0.2 μ L of sample from vial and run the programme. Based on the measured concentration trend of samples from 0 days to up to 9 month the reduction of antioxidant would be evaluated.

3.2.7.3.1 Evaluation of developed rapid color-based disc sensor in market ghee samples

Total 6 branded and unbranded ghee samples were collected from local market of karnal, Haryana. Antioxidant would be extracted as per the developed protocol under section **3.2.3.2**. The extract would be than used to check the sensitivity of the developed disc sensor.

3.2.7.3.2 Validation of developed disc sensor by comparing RP-TLC method to detect vegetable oil adulteration in ghee

Validation of developed disc sensor was done by using RP-TLC method (Rani *et al.*, 2015) to detect vegetable oil adulteration in 6 branded and 6 unbranded ghee samples collected from local market of karnal, Haryana.

3.2.8 Statistical analysis

The data obtained in the present study was subjected to one way and two-way Analysis of variance (ANOVA) for the significant difference in the means via Duncan's Multiple Comparison test performed at 95% confidence interval with IBM SPSS Statistics (version 20). The results were expressed as mean with standard deviation. The effect of storage days treatments and their combined effect on peroxide value, thiobarbituric acid value of ghee were analysed using factorial design of SPSS.

CHAPTER - 4

Results and Discussion

Results and Discussion

The main objective of the present study was to evaluate the vegetable oil adulteration in ghee on the basis of tracing of synthetic antioxidants (BHA and TBHQ) permitted in refined vegetable oils as per FSSAI. This chapter deals with the results obtained in the present study. This section has been discussed as per the order of the objectives of the study. The first objective was to develop color sensor for synthetic antioxidant detection. To accomplish this objective, (i) The concentration of DPPH and CeO_2 was standardized for efficient visual color change in the presence of pure antioxidants solution. (ii) Conditions for the efficient extraction of antioxidants from ghee samples were standardized (iii) color sensor based rapid method was standardized to detect the addition of refined vegetable oils in ghee.

In the second objective storage stability of developed color-based sensor was evaluated. GLC conditions were also standardized to evaluate the degradation of added antioxidants in stored ghee samples.

The third objective was designed for evaluation of developed rapid color-based sensor (Tube/Card/Disc) in stored and market ghee sample. To accomplish this objective, (i) color-sensor (Tube/Card/Disc) were evaluated in the stored ghee samples. (ii) Degradation of added antioxidants in stored ghee samples was checked using standardized GLC method. (iii) The developed color sensor's efficiency was evaluated in market ghee samples and results were compared with another method i.e., RP-TLC method which works on the detection of β -sitosterol, a sterol of plant origin and thereby checks the addition of vegetable oils in ghee.

4.1 DEVELOPMENT OF color SENSOR FOR SYNTHETIC ANTIOXIDANT DETECTION

This experiment was carried out to optimize the concentration of DPPH and CeO_2 so that a perceptible change in color is observed in the presence of standard synthetic antioxidants e.g., BHA, TBHQ, AsP etc. These results have been discussed under the following sub-heads

4.1.1 Optimization of dye (DPPH and CeO_2) concentration for efficient visual color change in presence of antioxidants

4.1.1.1: Optimization of DPPH concentration

DPPH is readily soluble in methanol and acetonitrile but for optimization of DPPH concentration, a solution (1, 2, 3 and 4 mM) concentration of DPPH was prepared in methanol. The reason was the lower boiling point of methanol at room temperature than acetonitrile. This unique property of methanol was further utilized in the preparation of paper based color sensor. Literature also suggested that methanol had been used as solvent for DPPH solution in paper-based techniques (Akar *et al.*, 2017; Sirivibulkovit *et al.*, 2018; Kuswandi *et al.*, 2020). s.

Solution of standard antioxidant BHA and TBHQ in acetonitrile was used in the study. The Ascorbyl palmitate (AsP) was prepared in ethanol. For optimization of DPPH concentration and volume, fixed volume of antioxidant solution i.e., 600 μL of minimum concentration (0.0025%) in acetonitrile was used. It is evident from the results (Fig 4.1) that in control i.e., acetonitrile without any antioxidant there was violet color on addition of DPPH solution 4mM (20 and 25 μL) displayed violet color. It is also evident from the results depicted in Fig 4.1 that a sharp yellow color appeared on addition of 20 & 25 μL of DPPH solution of 4 mM concentration in the antioxidant solution. This gave the clear-cut indication that either of the volumes of 4 mM DPPH solution can be used to bring a perceptible shift in color from violet to yellow. This was further confirmed from the findings (Fig 4.2) that 20 μL of 4mM DPPH was sufficient to show a color change from violet to yellow or disappearance of violet color in the presence of 600 μL of antioxidant solution of studied concentrations (0.0025 – 0.02%) in acetonitrile.

The response time required to shift the color from violet to yellow or disappearance of violet color of DPPH solution in the presence of different antioxidants have been depicted in table 4.1.

It is evident from the data (Table 4.1) that the response time (Min) to change color in the studied concentration (0.02, 0.01, 0.005 and 0.0025) % of BHA, TBHQ and AsP was (3.00 ± 0.50 , 6.33 ± 0.50 , 13.00 ± 1.00 , 26.77 ± 1.50), (1.00 ± 0.25 , 2.00 ± 0.50 , 3.00 ± 0.50 , 5.83 ± 1.00) and (0.50 , 1.00 ± 0.25 , 1.50 ± 0.50 and 2.0 ± 0.50), respectively. The response time of control one without any synthetic antioxidant gave more than 4400 min.

The optimized concentration of DPPH was also studied in other antioxidants like Gallic acid (GA), ascorbic acid (AA) and ascorbyl palmitate (AsP) @ 0.02, 0.03 and 0.05, respectively, the

level permissible in oils (FSSAI, 2017). It is evident from the results that DPPH color changed from

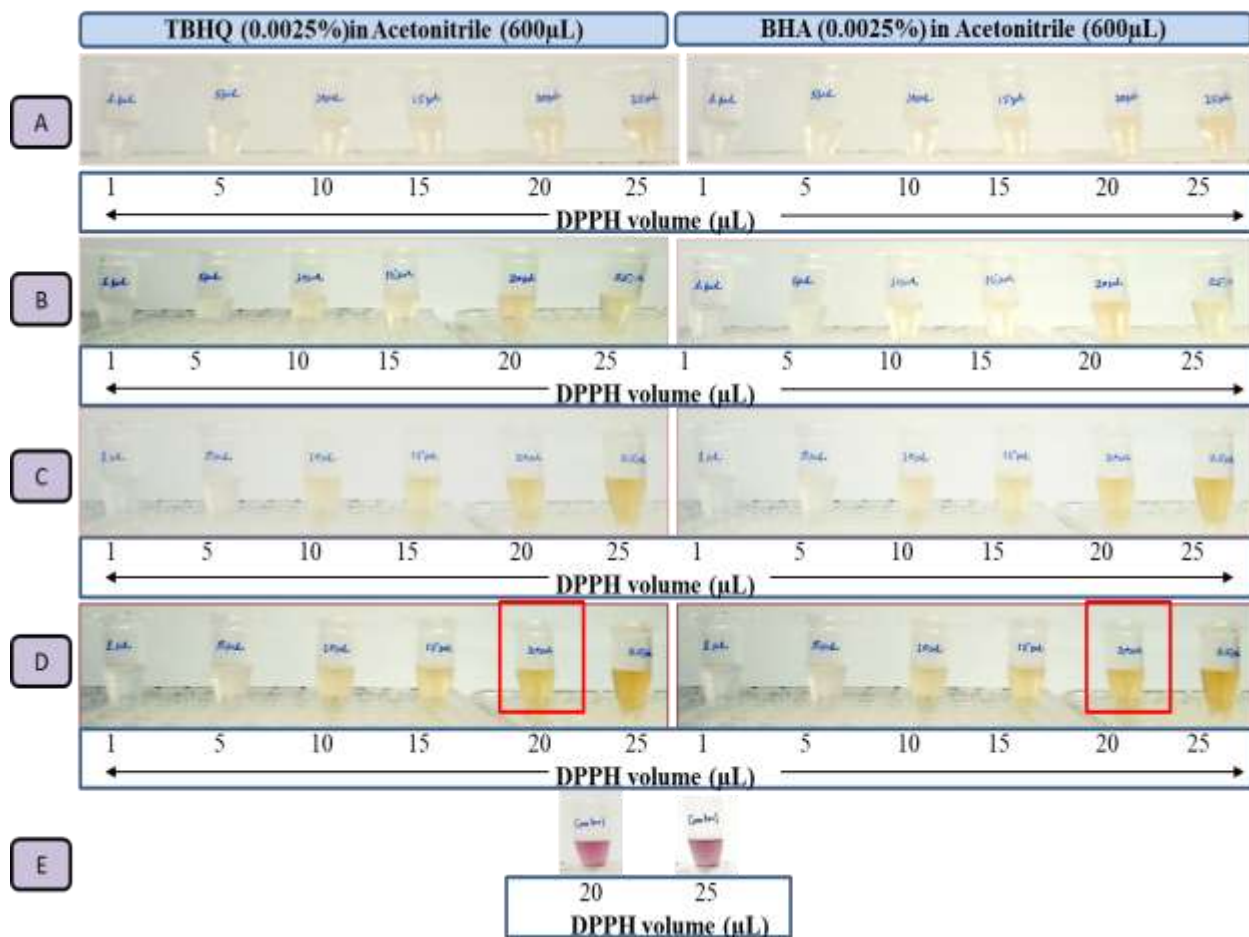


Figure 4.1: color change in DPPH solution in the presence of antioxidants (BHA and TBHQ) A: 1mM DPPH solution, B: 2mM DPPH solution, C: 3mM DPPH solution and D: 4mM DPPH solution E: Control; 4mM DPPH solution

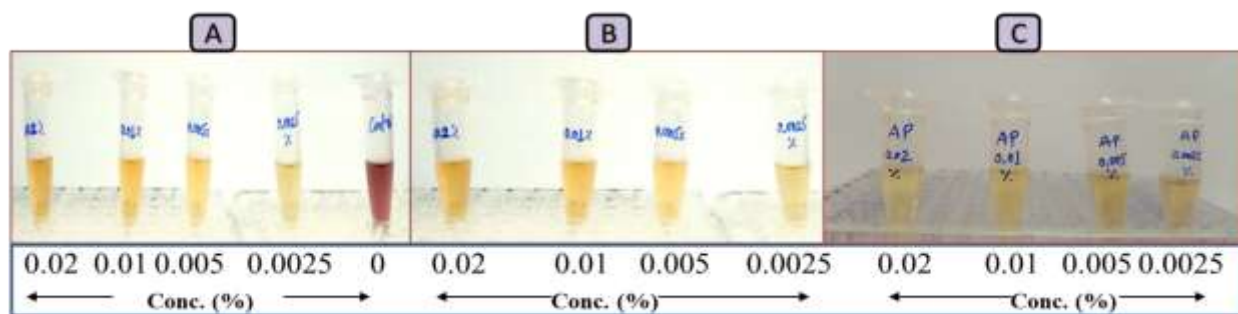


Figure 4.2: color change in DPPH solution (4mM) and volume (20 μL) in the presence of antioxidants solutions A: TBHQ solution in acetonitrile (600 μL), B: BHA solution in acetonitrile (600 μL), C: AsP solution in ethanol (600 μL)

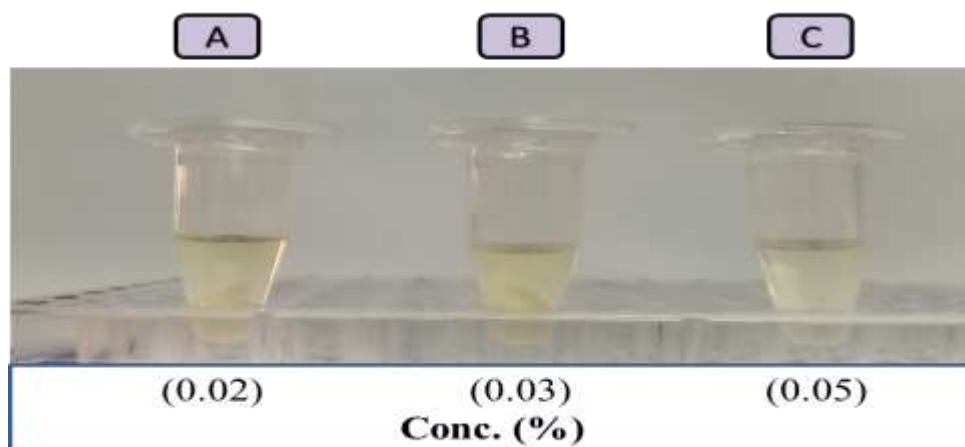


Figure 4.3: color change in DPPH solution (4mM) and volume (20 μ L) in the presence of antioxidants solutions A: GA solution in acetonitrile (600 μ L), B: AA solution in ethanol (600 μ L) and C: AsP solution in ethanol (600 μ L)

Table 4.1: Effect of antioxidant concentration on time of color change from violet to yellow after adding optimized volume of DPPH solution of 4 mM concentration

Concentration of antioxidant (%)	Time (Min)		
	BHA solution in acetonitrile	TBHQ solution in acetonitrile	AsP solution in ethanol
0	>4400	>4400	>4400
0.02	3.00 ± 0.50 ^A	1.00 ± 0.25 ^A	0.50 ± 0.00 ^A
0.01	6.33 ± 0.50 ^B	2.00 ± 0.50 ^B	1.00 ± 0.25 ^B
0.005	13.00 ± 1.0 ^C	3.00 ± 0.50 ^C	1.50 ± 0.25 ^C
0.0025	26.77 ± 1.5 ^D	5.83 ± 1.00 ^D	2.00 ± 0.50 ^D

Values are represented as Mean ± SD, n=3; ^{A-D} – different superscript column-wise differ significantly (p<0.05)

violet to light yellow (Fig 4.3). This indicated that DPPH solution of standardized concentration and volume can be used to detect the presence of these antioxidants and thereby the possibility of detecting vegetable oils in ghee. Earlier workers determined the capacity of DPPH in quenching the free radical in oils before and after test of accelerated oxidation. They used 0.2 ml of vegetable oils sample and 1 ml of DPPH (6.09×10^{-5} mol/liter) concentration and the absorbance was measured after 10 min at wavelength 520 nm. The disappearance of the radical followed a double-exponential equation in the existence of oils and oil fractions, which showed the existence of two (fast and slow) group of antioxidants. RSC was studied for the methanol-soluble phase ("methanolic fraction", MF) of the oil, the fraction non-soluble in methanol ("lipidic fraction", LF), and the non-fractionated oil ("total oil"; TF = MF + LF). Only rapeseed, linseed, sesame, safflower, and walnut oils showed significant RSC in the MF owing to the presence of phenolic compounds. (Espin *et. al.*, 2000). It was reported that color of DPPH was reduced in less than 10 min.

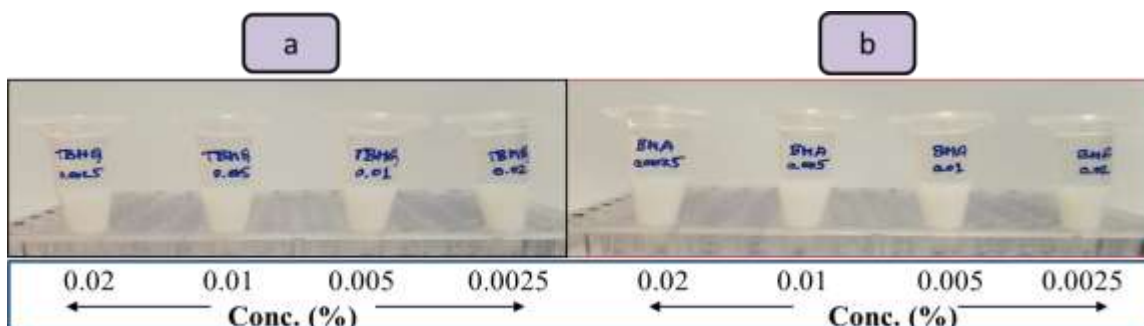
Ramani *et al.*, (2018) also reported that the color of DPPH changed from violet to yellow color by adding palm oil adulterated ghee up to 5% within 30 min. In case of pure ghee, the color remained violet. Cotton seed oil adulterated ghee samples also changed DPPH color from violet to yellow within 10 min at 5% level (Joshi, 2015).

Kuswandi (2020) also used DPPH and optimized 5 mM concentration for detection of antioxidant (GA) in beverages and herbal products and observed the color change from violet to yellow color. The same color change was also observed in case of antioxidant AA, GA, BHT etc. (Akar *et al.*, 2017; Sirivibulkovit *et al.*, 2018)

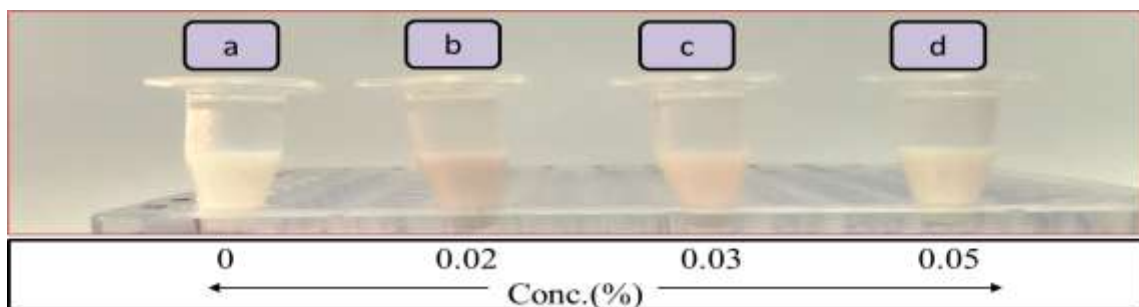
4.1.1.2 Optimization of cerium oxide (CeO₂) colloidal dispersion in 20% acetic acid concentration

In this experiment, 20 μ L of CeO₂ colloidal dispersion in 20% acetic acid were added into 600 μ L of standard antioxidants solutions in acetonitrile for BHA (0.02%), TBHQ (0.02%), and GA (0.02%), in ethanol for AA (0.03%) and Asp (0.05%). It is evident from the results (Fig 4.4) that in case of BHA and TBHQ, there was no color changes but in case of antioxidants like GA, AA and AsP there was a change in color from white to yellowish or brownish (Fig 4.4). This can be attributed to the fact that GA, AA and AsP have ability to chelate metal ions which was responsible for showing a color change (Sharp *et al.*, 2012& 2014). However, the other two antioxidants (BHA and TBHQ) lack the ability to chelate metal ions and therefore could not bring a change in color. The color concentration/intensity was varied for the types of antioxidants, reducing capacity of Ce⁴ to Ce³ and binding ability to the ceria surface. The response of the sensors fabricated using CeO₂ were directly proportional to the oxidation potential and the chemical structure of antioxidants like size, position (-*ortho*) and number of OH substituents (Sharp *et al.*, 2012& 2014). In another experiment CeO₂ was impregnated on filter paper to see the effect of antioxidants on dried cerium oxide. On adding 20 μ L of the above said antioxidants (0.02, 0.01, 0.005 and 0.0025%) solutions were added and the color change on the CeO₂ impregnated paper was observed. It is evident from the results (Fig 4.5) that BHA and TBHQ could not bring any change in color whereas AA (1, 2, 3, 4, 5, 10) mM, GA (0.5, 0.1, 1.5 and 2.0) mM and AsP (1, 2, 4, 6, 8, 10) mM, were able to bring a change in color and intensity of color change increased as the concentrations of AA, GA and AsP was increased.

From the above discussion it was concluded that out of the two studied materials i.e., DPPH and CeO₂, the DPPH was found to be more useful because of its ability to respond to all the studied antioxidants and their different concentrations. Therefore, DPPH was selected in further study.



(A)



(B)

Fig 4.4 Effect of antioxidants solution on the color development in 20 μ L of Cerium Oxide 20% dispersion solution: (A) TBHQ in acetonitrile (600 μ L) & BHA in acetonitrile (600 μ L) and (B) a: Control, b:GA in acetonitrile (600 μ L), c: AA in ethanol (600 μ L) & d: Asp in ethanol (600 μ L)

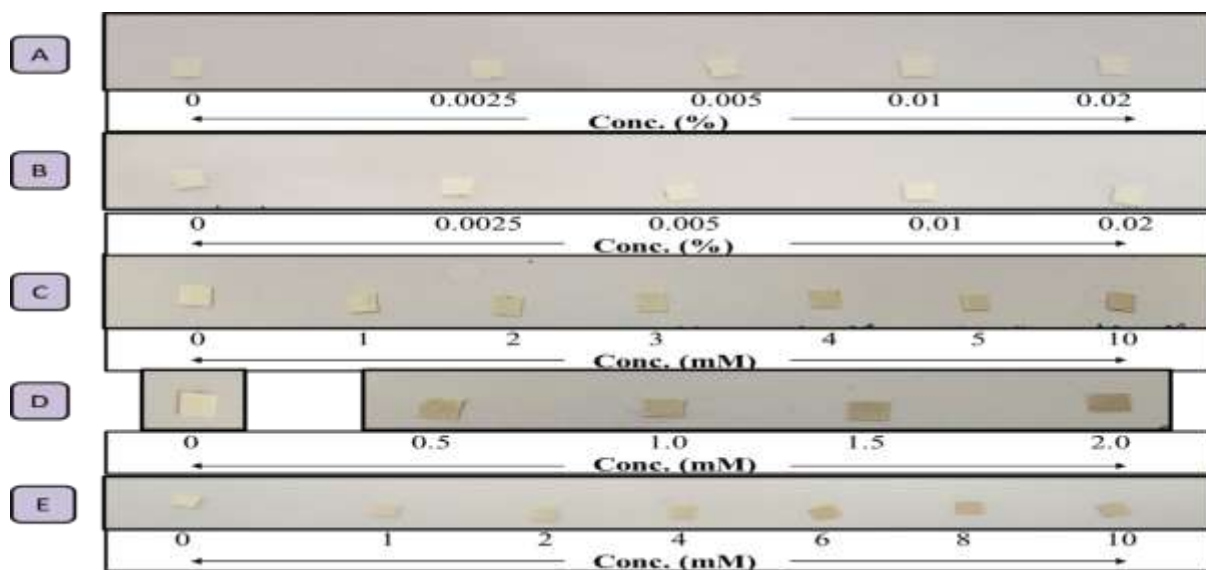


Fig 4.5 color development in Cerium Oxide impregnated dried paper disc in the presence of (A) BHA, (B) TBHQ, (C) Ascorbic Acid (D) Gallic Acid and (E) Ascorbyl Palmitate

4.1.2 Optimization of conditions for the efficient extraction of antioxidants from ghee samples

To optimize the conditions for efficient extraction of added antioxidants from ghee, two approaches were evaluated. First was the single step extraction using hexane and acetonitrile as mentioned in 3.2.3.2 section of material & methods. It is evident from the results (Table 4.2), wherein that time taken to change the color of DPPH solution varied from about 3 – 1 min, depending upon the concentration of BHA present in the ghee samples. As the concentration of added BHA in ghee was decreased the time required to see the perceptible change in color of DPPH solution from violet to yellow / colorless was increased. It is also evident from the results that there was a significant ($p \leq 0.05$) difference in the increase of time as the concentration of the added BHA was decreased. In case of TBHQ, it was observed that the time required to observe the color change was about 1 min up to the added concentration of BHA to the tune of 0.005 to 0.02%. However, a significant increase in the time to change the color was observed in samples containing 0.0025% of TBHQ. Almost similar trend was observed in case of ascorbyl plamate containing samples. It is clearly evident from the results that in case of control ghee samples i.e., without any added antioxidant, the time required to change the color of DPPH solution varied from (82 ± 3.00 to 86 ± 4.00 min) antioxidant added/spiked ghee samples.

Table 4.2 Effect of single step antioxidant extraction from ghee on the time of color change of DPPH solution

Concentration of antioxidant added in ghee (%)	Antioxidant in acetonitrile extract		
	BHA (600 μ L)	TBHQ (600 μ L)	AsP (600 μ L)
	----- Time (min)-----		
0	86 ± 4.00^E	82 ± 3.00^E	85 ± 3.50^E
0.02	1.00 ± 0.25^A	1.00 ± 0.25^A	0.25 ± 0.25^A
0.01	1.83 ± 0.29^B	1.00 ± 0.29^A	0.25 ± 0.25^A
0.005	2.50 ± 0.5^C	1.00 ± 0.50^A	0.25 ± 0.25^A
0.0025	3.00 ± 0.50^D	1.83 ± 0.50^B	0.75 ± 0.25^B

Values are represented as Mean $\pm$ SD, n=3; ^{A-E} – different superscript column-wise differ significantly ($p < 0.05$)

In case of two steps extraction, where in extraction of the sample was again performed using hexane and acetonitrile with additional step of second extraction using acetonitrile. The details have been discussed in section 3.2.3.2. It is evident from the results (Table 4.3) that observed time of color change in DPPH solution was found to be more in all the studied concentrations of added antioxidants in ghee. The time varied from 3- 27 min in case of BHA depending upon the concentration of the antioxidant added to ghee samples.

Table 4.3 Effect of two steps antioxidant extraction from ghee the time of color change of DPPH solution

Concentration of antioxidant added in ghee (%)	Antioxidant in acetonitrile extract		
	BHA (~2000µL)	TBHQ (~2000µL)	AsP (~2000µL)
	----- Time (min)-----		
0	240± 7 ^E	243± 9 ^E	245± 12 ^E
0.02	3.00± 0.25 ^A	1.00 ^A	0.50 ^A
0.01	6.00± 0.50 ^B	2.00± 0.50 ^B	1.00± 0.25 ^B
0.005	12.66± 1.00 ^C	3.00± 0.50 ^C	1.50± 0.25 ^C
0.0025	27.00± 1.50 ^D	6.00± 0.50 ^D	2.00± 0.500 ^D

Values are represented as Mean ± SD, n=3; ^{A-E} – different superscript column-wise differ significantly (p<0.05)

Similarly, in case of TBHQ and ascorbyl palmitate to the increase in time to change the color was observed in comparison to single step extraction. This increase in time to change to color of DPPH solution could be attributed to the effect of dilution, wherein in extracted antioxidants in two step extraction approach were present in about 2 ml of acetonitrile where as in single step these were extracted in about 1 ml of acetonitrile. The double extraction was selected over single extraction because of significant differences of color change time of DPPH in each antioxidant concentrations. It is also evident from the color (Fig 4.6) that after the double extraction the difference in color was clearly visible in the extract obtained from control (violet) versus extract obtained from ghee samples containing antioxidants (BHA and TBHQ) (yellow).

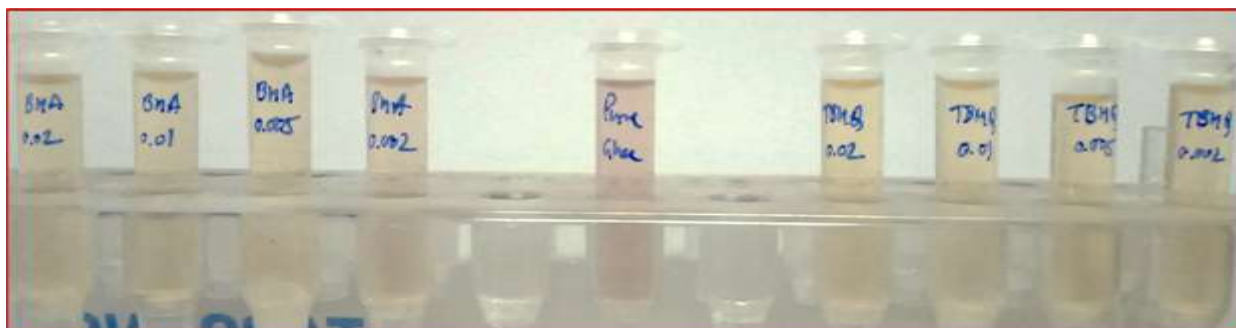


Fig 4.6 color change in acetonitrile extract on adding DPPH solution

4.1.3 Development of color sensor based rapid method on the basis of selected detecting agent

4.1.3.1 Fabrication of card disc and tube sensor

The card, disc and tube sensors were fabricated as described in section 3.2.4 of material and methods.

4.1.3.2 Optimization of DPPH on card, disc and in tube sensor

4.1.3.2.1 Optimization of DPPH on card sensor

Optimization of DPPH on card sensor it is evident from the result (Fig 4.7) that card sensor prepared by impregnating with 1 μ L and 5 μ L DPPH solution of 4mM, exhibited a small color change on adding 20 μ L of 0.0025% antioxidant solution, but this change was not sharp. Since, the card sensor did not work at the lowest concentration of antioxidant which is expected in ghee samples having 1% refine vegetable oils Therefore, the developed card sensor was not further used in the study.

4.1.3.2.2 Optimization of DPPH on disc sensor

For optimization of disc sensor, DPPH solution of 4 mM concentration was impregnated on paper discs by adding different volume 1, 5, 10, 15, 20 and 25 μ L of this solution. These developed discs were then added into 600 μ L 0.0025% solution of antioxidants i.e., TBHQ, BHA and AsP and observed the color change through naked eyes. The result showed (Fig 4.8) showed that disc prepared by adding 20 μ L of DPPH solution resulted into a sharp color change from violet to yellow in the antioxidant containing tubes.

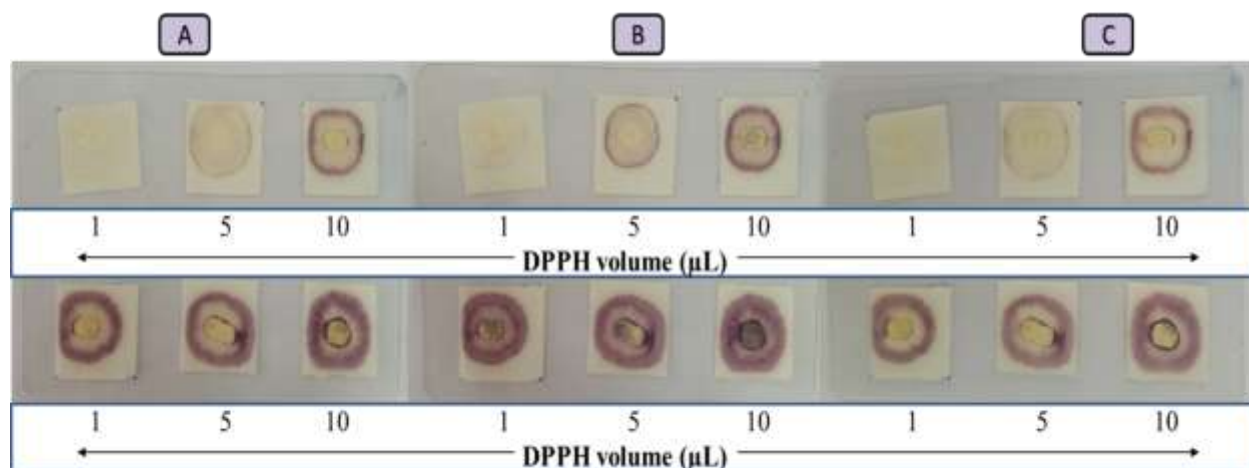


Fig. 4.7: A: 20 μL of TBHQ solution in acetonitrile (0.0025%) , B: 20 μL BHA solution in acetonitrile (0.0025%) and C: 20 μL of AsP solution in ethanol (0.0025% μL)

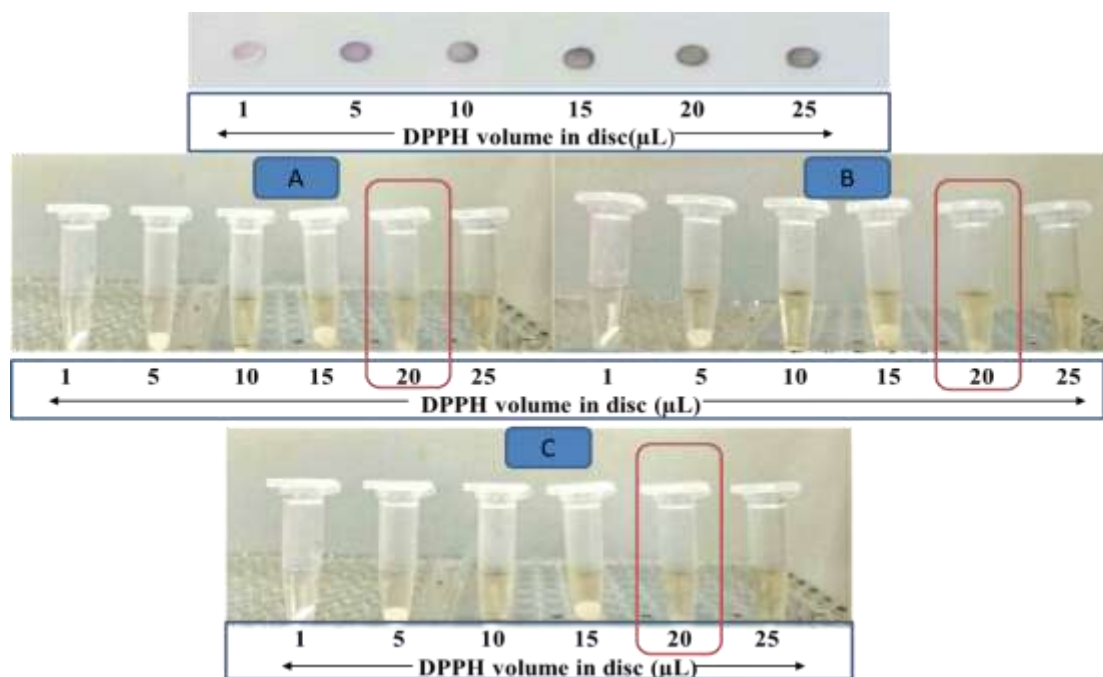


Fig. 4.8: A: TBHQ solution (0.0025%) in acetonitrile (600 μL), B: BHA solution (0.0025%) in acetonitrile (600 μL), C: Ascorbyl palmitate (0.0025%) solution in ethanol (600 μL)

4.1.3.2.3 Effect of antioxidants concentration on the response of developed disc-based sensor

The result obtained from the disc sensor are presented in Table 4.4 and Fig 4.9, it was observed that there was a significant difference ($P < 0.05$) of timing of color change from violet to yellow. The response time of control sample was more than 4400 min. The response time also varied with the concentration of antioxidant as well as type of antioxidant. In case of BHA solution the response time was slightly more than the time required in TBHQ and ascorbyl palmitate. The time observed was almost similar to the experiment carried out using liquid DPPH of same concentration. The advantage of the developed disc sensor was its ease of use and no botheration of preparation of any liquid dye.

Table 4.4 Effect of antioxidants concentration on the response of developed disc-based sensor

Concentration of antioxidants (%)	Time (min)		
	BHA solution in acetonitrile (600 μ L)	TBHQ solution in acetonitrile (600 μ L)	AsP solution in ethanol (600 μ L)
0	>4400	>4400	>4400
0.02	3.00 $\pm$ 0.50 ^A	1.00 $\pm$ 0.00 ^A	0.50 $\pm$ 0.00 ^A
0.01	6.33 $\pm$ 0.53 ^B	2.00 $\pm$ 0.025 ^B	1.00 $\pm$ 0.25 ^B
0.005	12.67 $\pm$ 1.33 ^C	3.00 $\pm$ 0.50 ^C	1.50 $\pm$ 0.50 ^C
0.0025	27.00 $\pm$ 1.58 ^D	6.33 $\pm$ 0.50 ^D	2.00 $\pm$ 0.50 ^D

Values are represented as Mean $\pm$ SD, n=3; ^{A-D} – different superscript column-wise differ significantly ($p < 0.05$)

4.1.3.2.4 Effect of antioxidants concentration on the response of developed tube-based sensor

The result obtained from the tube sensor are presented in Table 4.5 and Fig 4.10, it was observed that there was significant difference ($P < 0.05$) of timing in changing the color of DPPH from violet to yellow among the various concentrations of each antioxidant (TBHQ, BHA and AsP) of 600 μ L into the dry DPPH tubes. The response time of control sample was more than 4400 min. The tube sensor worked well with good response of color change same as optimized as liquid DPPH for above antioxidants.

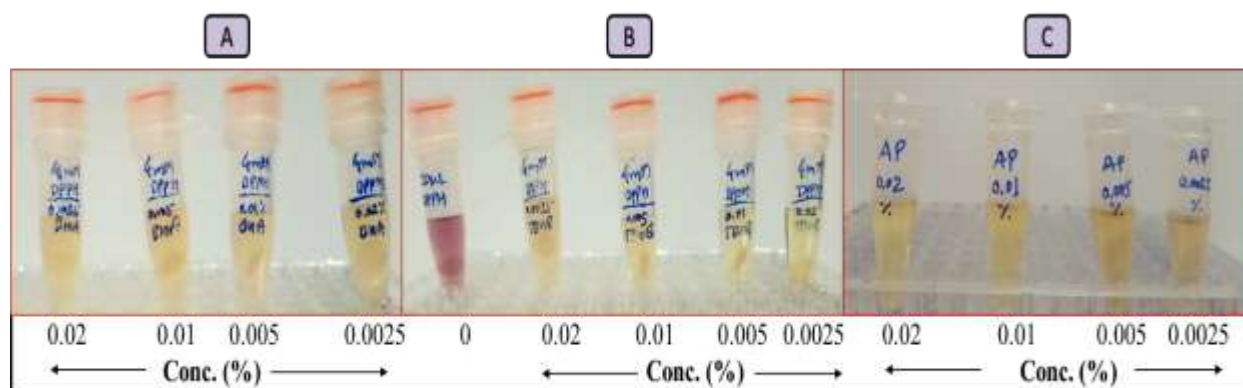


Fig. 4.9: Effect of antioxidants' concentration on the response of developed disc-based sensor A: TBHQ solution in acetonitrile (600 µL), B: BHA solution in ethanol (600 µL) and C: AsP solution in ethanol (600 µL)

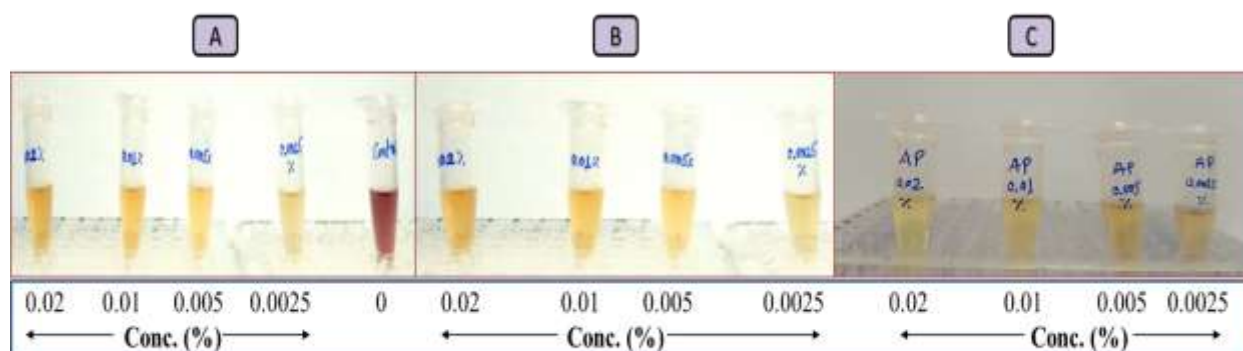


Fig. 4.10: Effect of antioxidants concentration on the response of developed tube-based sensor A: TBHQ solution in acetonitrile (600 µL), B: BHA solution in ethanol (600 µL) and C: AsP solution in ethanol (600 µL)

Table 4.5 Effect of antioxidants concentration on the response of developed tube-based sensor

Concentration of antioxidants (%)	Time (min)		
	BHA solution in acetonitrile (600μL)	TBHQ solution in acetonitrile (600μL)	AsP solution in ethanol(600μL)
0	>4400	>4400	>4400
0.02	3.00 ± 0.25 ^A	1.00 ^A	0.50 ^A
0.01	6.53 ± 0.50 ^B	2.00± 0.25 ^B	1.00± 0.25 ^B
0.005	13.00 ± 1.25 ^C	3.00± 0.50 ^C	1.50± 0.25 ^C
0.0025	27.25 ± 1.50 ^D	6.00± 0.50 ^D	2.25± 0.50 ^D

Values are represented as Mean ± SD, n=3; ^{A-D} – different superscript column-wise differ non significantly (p>0.05)

4.2 Storage stability of developed color sensors (tube and disc sensors)

Tube sensors packed in both aluminum foil and cardboard box were evaluated for their storage stability at ambient temperature (30°C) and refrigerated temperature (4-6°C). In case of disc sensors, the prepared paper -based disc sensors were packed in HDPE amber colored bottle and clear plastic bottles and stored at ambient temperature (30°C) and refrigerated temperature (4-6°C). To check the effect of storage of sensors, the response time to change color in the antioxidant solutions of BHA and TBHQ of variable concentration (0.02, 0.01, 0.005 and 0.0025%) was measured at 15 days interval.

4.2.1 Storage stability of tube sensor at ambient temperature (30° C)

It is clearly evident from the results (Table 4.6 and Fig 4.11) that the response time of the 15 days stored tube sensor was close to the response time of the sensor to the same concentration of the antioxidant on zero day of storage. This confirmed that the tube sensor stored at ambient temperature remained stable up to 15 days only. Kuswandi *et al.*, (2020) also reported that the paper microzone plate-based DPPH was stable only 5 hours at room temperature (25°C). The less shelf life was due the absence of any lamination or packaging.

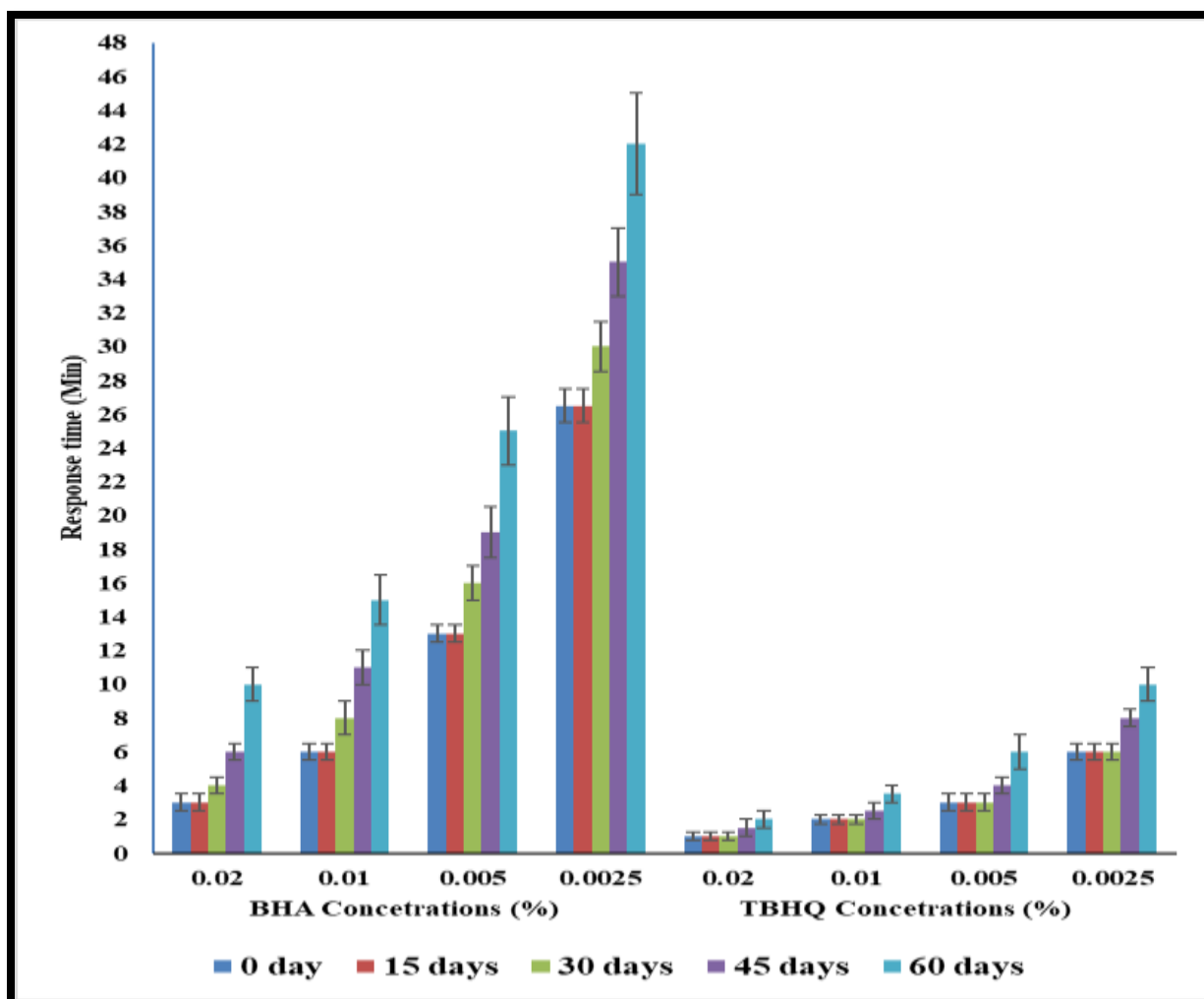


Fig 4.11 Storage stability of tube sensor at ambient temperature (30° C)

Table 4.6 Storage stability of tube sensor at ambient temperature (30° C)

Storage period (days)	BHA conc (%)				TBHQ conc (%)			
	0.02	0.01	0.005	0.0025	0.02	0.01	0.005	0.0025
	----- Time (min)-----							
0	3±0.50 ^a	6±0.50 ^a	13±0.5 ^a	26.5±1.0 ^a	1±0.25 ^a	2±0.25 ^a	3±0.50 ^a	6±0.50 ^a
15	3±0.50 ^a	6±0.50 ^a	13±0.5 ^a	26.5±1.0 ^a	1±0.25 ^a	2±0.25 ^a	3±0.50 ^a	6±0.50 ^a
30	4±0.50 ^b	8±1.0 ^b	16±1.0 ^b	30±1.5 ^b	1±0.25 ^a	2±0.25 ^a	3±0.50 ^a	6±0.50 ^a
45	6±0.50 ^c	11±1.0 ^c	19±1.5 ^c	35±2.0 ^c	1.5±0.50 ^b	2.5±0.5 ^b	4±0.50 ^b	8±0.50 ^b
60	10±1.0 ^d	15±1.5 ^d	25±2.0 ^d	42±3.0 ^d	2±0.50 ^c	3.5±0.5 ^c	6±1.0 ^c	10±1.0 ^c

Values are represented as Mean ± SD, n=3; ^{a-d} – different superscript column-wise differ significantly (p<0.05)

4.2.2 Storage stability of tube sensor at refrigerated temperature (4-6° C)

It is evident from the data (Table 4.7) that the response time of tube sensors stored at refrigerated temperature remained unaffected till 30th days of storage and their response time was close to the response time of zero-day stored sensor. The storage stability of the tube sensor was observed 30th days at refrigerated temperature(4-6°C). Kuswandi *et al.*, (2020) also reported that the paper microzone plate-based DPPH was stable at chiller condition (4°C) up to 3 days.

Table 4.7 Storage stability of tube sensor at refrigerated temperature (4-6° C)

Storage period (days)	BHA conc (%)				TBHQ conc (%)			
	0.02	0.01	0.005	0.0025	0.02	0.01	0.005	0.0025
	----- Time (min)-----							
0	3±0.5 ^a	6±0.5 ^a	13±0.5 ^a	26.5±1 ^a	1±0.25 ^a	2±0.25 ^a	3±0.5 ^a	6±0.5 ^a
15	3±0.5 ^a	6±0.5 ^a	13±0.5 ^a	27.0±1 ^a	1±0.25 ^a	2±0.25 ^a	3±0.5 ^a	6±0.5 ^a
30	3±0.5 ^b	6±1 ^b	13±1 ^b	27±1.5 ^b	1±0.25 ^a	2±0.25 ^a	3±0.5 ^a	6±0.5 ^a
45	5±0.5 ^c	8±1 ^c	17±1.5 ^c	34±2 ^c	1±0.5 ^b	2±0.5 ^b	3±0.5 ^b	8±1.0 ^b
60	8±1 ^d	11±1.5 ^d	24±2 ^d	46±3 ^d	2±0.5 ^c	3.5±1 ^c	7±1 ^c	11±1.5 ^c

Values are represented as Mean ± SD, n=3; ^{a-d} – different superscript column-wise differ significantly (p<0.05)

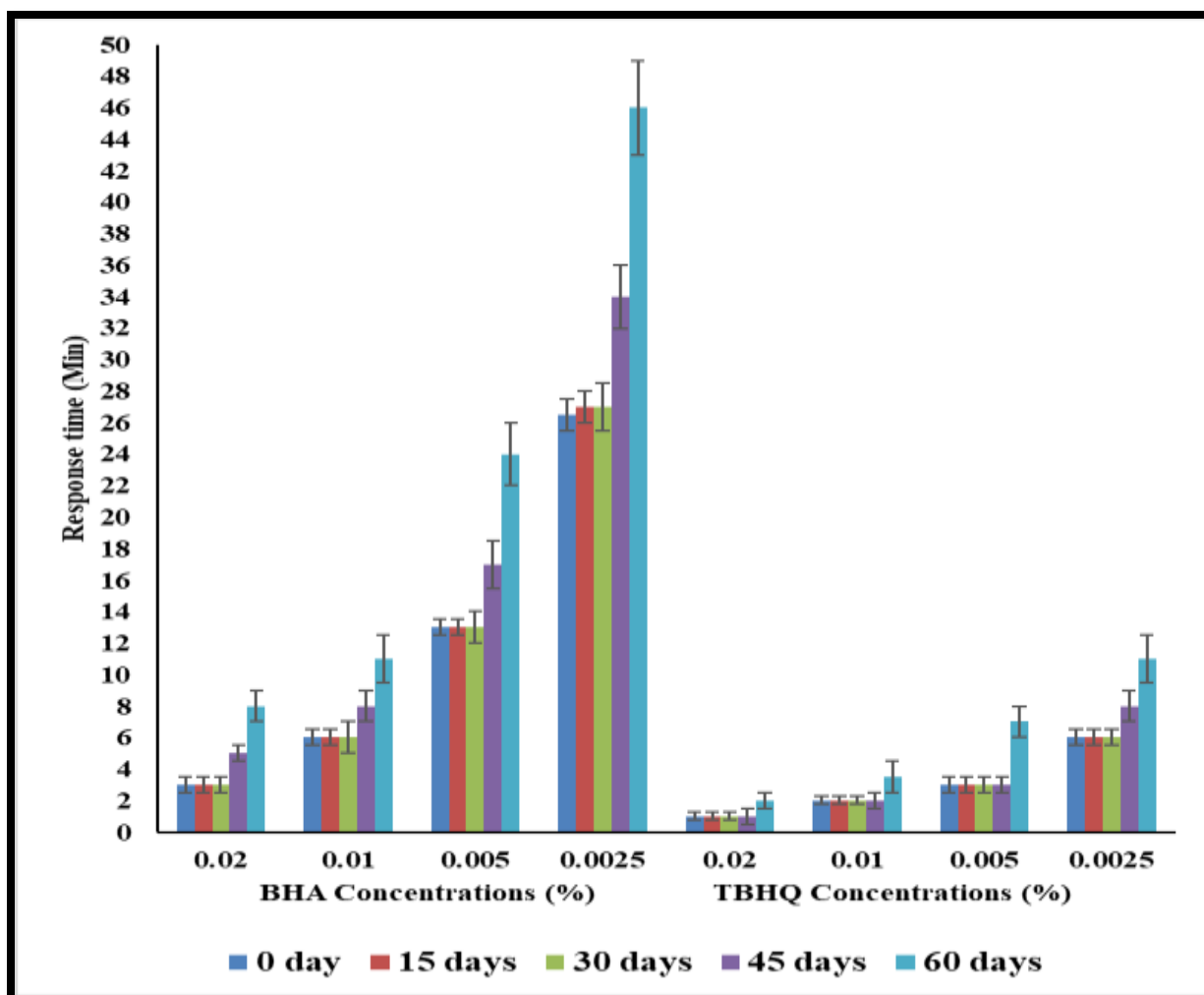


Fig 4.12 Storage stability of tube sensor at refrigerated temperature (4-6°C)

4.2.3 Storage stability of disc sensor at ambient temperature (30° C)

The perusal of the data (Table 4.8 and Fig 4.13) revealed that the response time of the stored discs was close to the freshly prepared discs for the corresponding concentration of the pure antioxidant solution. These discs gave results comparable to freshly prepared discs till the 30th day of storage at ambient temperature (30°C) and both amber color and clear plastic bottles. The similar result reported that the paper-based DPPH device wrapped with aluminum foil was stable up to 30 days at room temperature (26-31°C) by (Sirivibulkovit *et al.*, 2018).

Table 4.8 Storage stability of disc sensor at ambient temperature (30° C)

Storage period (days)	BHA conc (%)				TBHQ conc (%)			
	0.02	0.01	0.005	0.0025	0.02	0.01	0.005	0.0025
	----- Time (min)-----							
0	3±0.5 ^a	6±0.5 ^a	13±0.5 ^a	26.5±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
15	3±0.5 ^a	6±0.5 ^a	13±0.5 ^a	26.5±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
30	3±0.5 ^a	6±0.5 ^a	13±0.5 ^a	27.0±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
45	5±0.5 ^b	8±1 ^b	16±1.5 ^b	30.0±2 ^b	2.0±0.5 ^b	3.5±0.5 ^b	5±0.5 ^b	8±1.0 ^b
60	8±1 ^c	11±1.5 ^c	21.6±2 ^c	34.0±3 ^c	3.0±0.5 ^c	5.0±1.0 ^c	7±1 ^c	11±1.5 ^c

Values are represented as Mean ± SD, n=3; ^{a-c} – different superscript row-wise differ significantly (p<0.05)

4.2.4 Storage stability of disc sensor at refrigerated temperature (4-6° C)

The perusal of the data (Table 4.9) revealed that there was no significant change in the response time till 90 days of storage at refrigerated temperature (4-6°C) and both amber color and clear plastic bottles. Thereafter, a significant (p≤ 0.05) increase in the response time of the stored sensors (Fig 4.14) was observed for all the three studied antioxidants and their corresponding concentrations. It was concluded that disc sensors were found to have better storage stability both at room as well as refrigerated temperature. Hence, these paper disc-based sensors with impregnated DPPH were finalized for further use in the course of the study. The findings of the present investigation were in accordance with the earlier reports (Sirivibulkovit *et al.*, 2018) that paper-based DPPH device wrapped with aluminum foil was stable up to 30 days at refrigerated temperature (2-4°C).

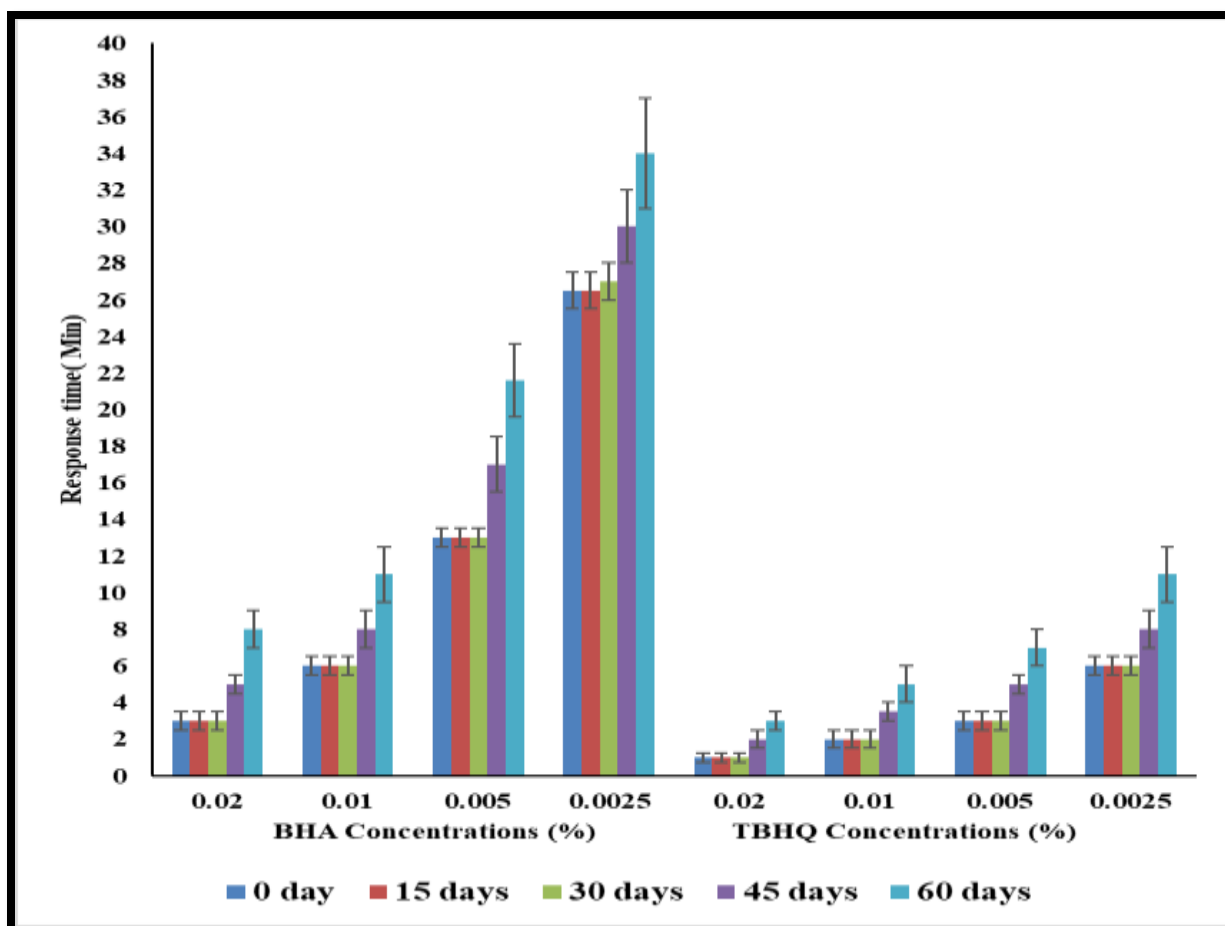


Fig 4.13 Storage stability of disc sensor at ambient temperature (30° C)

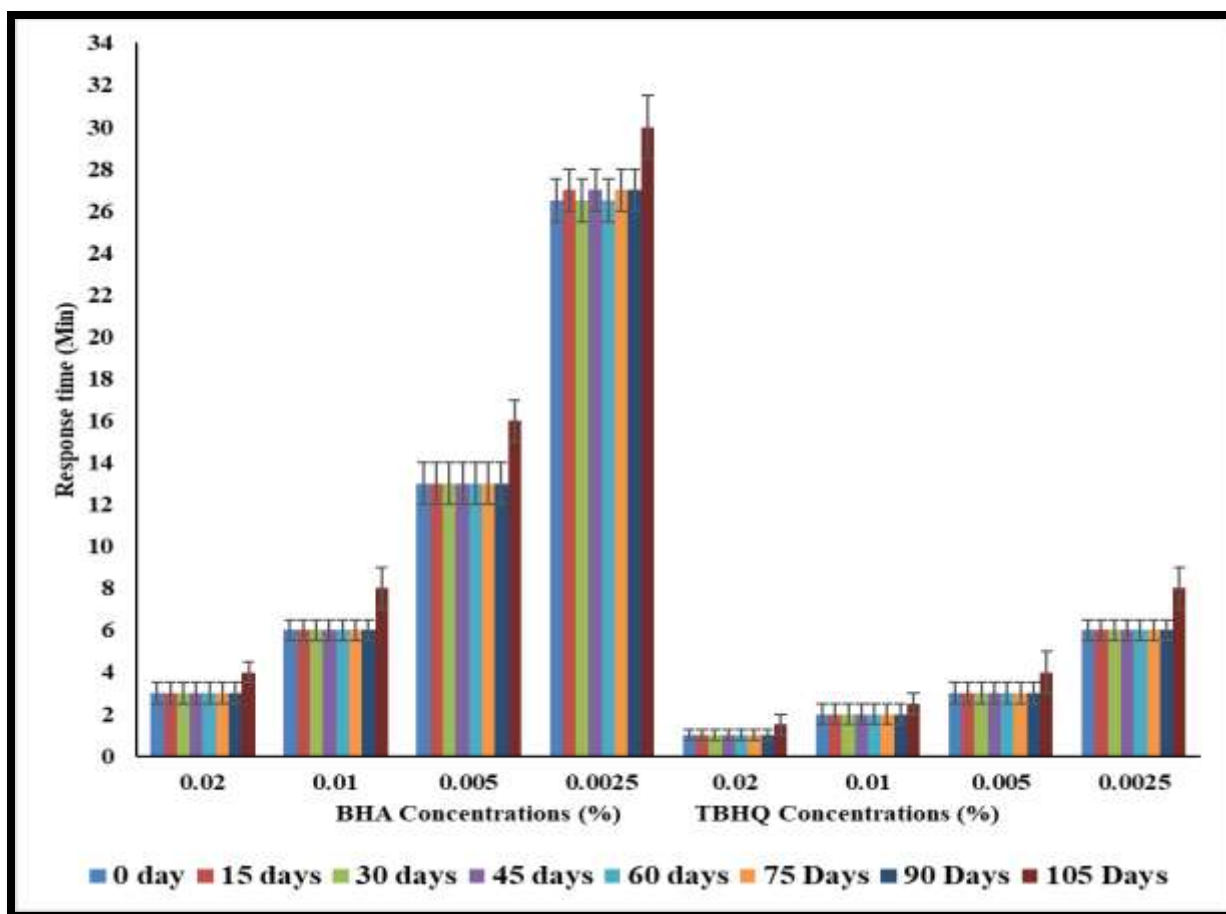


Fig 4.14 Storage stability of disc sensor at refrigerated temperature (4-6° C)

Table 4.9 Storage stability of Disc sensor at refrigerated temperature (4-6° C)

Storage period (days)	BHA conc (%)				TBHQ conc (%)			
	0.02	0.01	0.005	0.0025	0.02	0.01	0.005	0.0025
	----- Time (min)-----							
0	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	26.5±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
15	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	27.0±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
30	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	26.5±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
45	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	27.0±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
60	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	26.5±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
75	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	27.0±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
90	3±0.5 ^a	6±0.5 ^a	13±1.0 ^a	27.0±1 ^a	1±0.25 ^a	2±0.5 ^a	3±0.5 ^a	6±0.5 ^a
105	4±0.5 ^b	8±1.0 ^b	16±1.0 ^b	30±1.5 ^b	1.5±0.5 ^b	2.5±0.5 ^b	4±1.0 ^b	8±1.0 ^b
120	6±1.0 ^c	11±1.5 ^c	19±1.5 ^c	35±2 ^c	2±0.5 ^c	3.5±1.0 ^c	6±1.0 ^c	10±1.0 ^c

Values are represented as Mean ± SD, n=3; ^{a-c} – different superscript column-wise differ significantly (p<0.05)

4.2.5 Standardization of GLC conditions to evaluate the degradation of added antioxidants in stored ghee samples

The following two methods (i) specified for triglyceride analysis using column CP-7532 (5m x0.53mmx 0.17µm) as per the method described in ISO: 17678(2010) and (ii) SPB-5 (Supelco), (30m x0.53mmx1.5µm) column in the method as specified for synthetic antioxidant (BHA, BHT and TBHQ) detection in vegetable oils by Yang *et al.*, (2002) were standardized in the laboratory.

4.2.5.1 Resolution of standard antioxidants using the conditions specified for triglyceride analysis using column CP-7532 (5m x0.53mmx 0.17µm) as per the method described in ISO: 17678(2010)

For standardization of the first method the conditions as per the method described in ISO: 17678(2010) by using standard antioxidant BHA, BHT and TBHQ of minimum concentrations (0.0025%) in acetonitrile, separately and run the above standards in GLC condition as Table 3.3 under section 3.2.6.2 of material and methods. It is evident from the chromatogram (Fig 4.15) that BHA and BHT were eluted at the same retention time of 1.9 min. It is also clear from the chromatogram that TBHQ eluted at 2.46 min. This clearly revealed that the column used and conditions of oven temperature and carrier gas flow could not resolve the studied antioxidants

especially BHA and BHT. Therefore, these GLC conditions and column was not used in further study and another method was evaluated so as to resolve all the three studied antioxidants i.e. BHA, BHT and TBHQ.

4.2.5.2 Resolution of standard antioxidants using the conditions specified by Yang et al. (2002)

In this method, authors used SPB-5 (Supelco), (30m x0.53mmx1.5µm) column, hence same column was used in the present investigation and other conditions used have been specified in Materials & Methods section (3.2.6.2). It is clearly evident in the chromatogram (Fig 4.16), that BHA, BHT and TBHQ had retention time 7.45, 8.0 and 8.35 mins, respectively. This clearly showed that the conditions of GLC were able to resolve the antioxidants and method can be used to check the degradation of BHA or BHT in ghee samples spiked with vegetable oils and subsequently stored.

It is also evident from the standard curved prepared by spiking standard antioxidants in ghee (Fig 4.17) that the method was linear above 0.02% of standard antioxidant concentration of BHA and TBHQ. The R^2 was 0.9974 for BHA and 0.9817 for TBHQ.

It was contemplated that to evaluate the degradation of antioxidants in vegetable oils spiked ghee samples, the method should be workable in samples spiked with to at least 1% of vegetable oils. Therefore, the Standard curve was also prepared after the extraction of the antioxidants from ghee samples spiked with different concentrations of standard antioxidants as per the extraction method described in section 3.3.3.2. It is evident from the standard curves (Fig 4.18) the method was linear for both BHA and TBHQ up to 0.0025% concentration and above. R^2 for both the antioxidants was above 0.99. These findings clearly confirmed that the standardized method can be used to check the degradation of antioxidants in ghee samples spiked with refined vegetable oils @ minimum 1% and above.

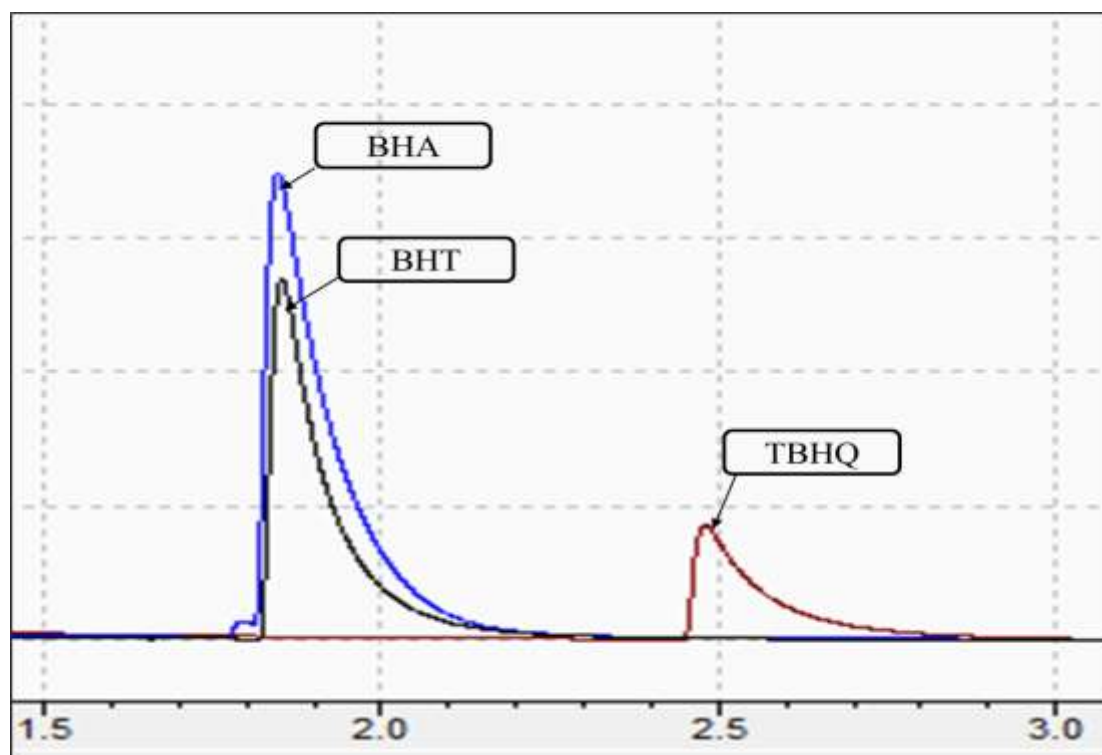
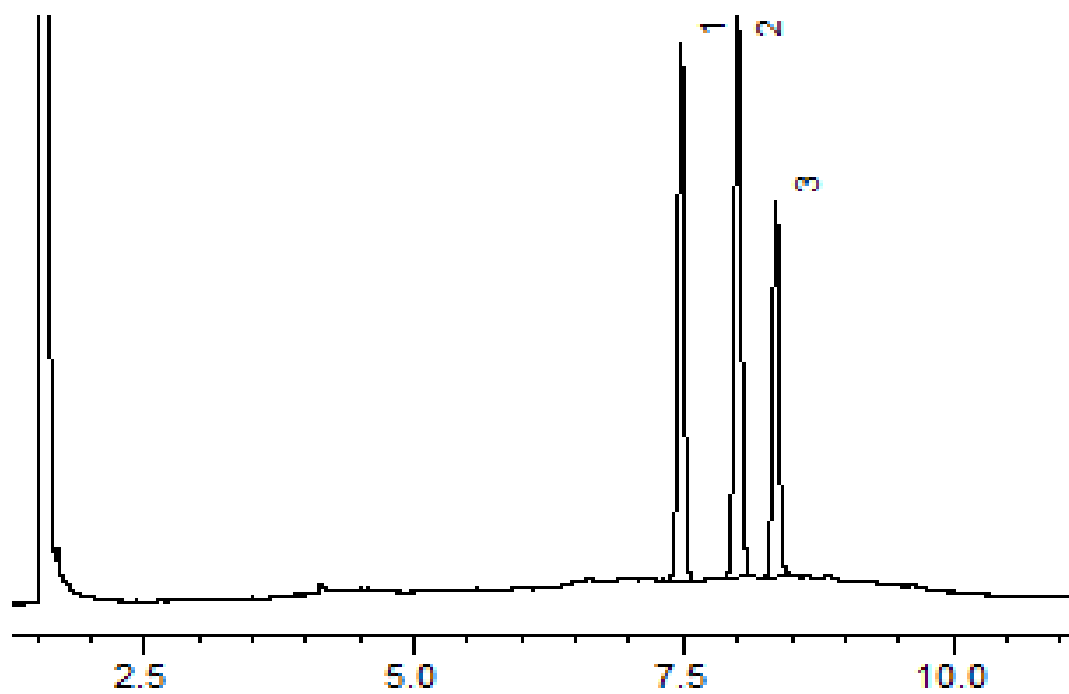


Fig 4.15 Chromatogram of standard antioxidant BHA, BHT and TBHQ



1: BHA; 2: BHT and 3: TBHQ

Fig 4.16 Chromatogram of BHA, BHT and TBHQ in acetonitrile

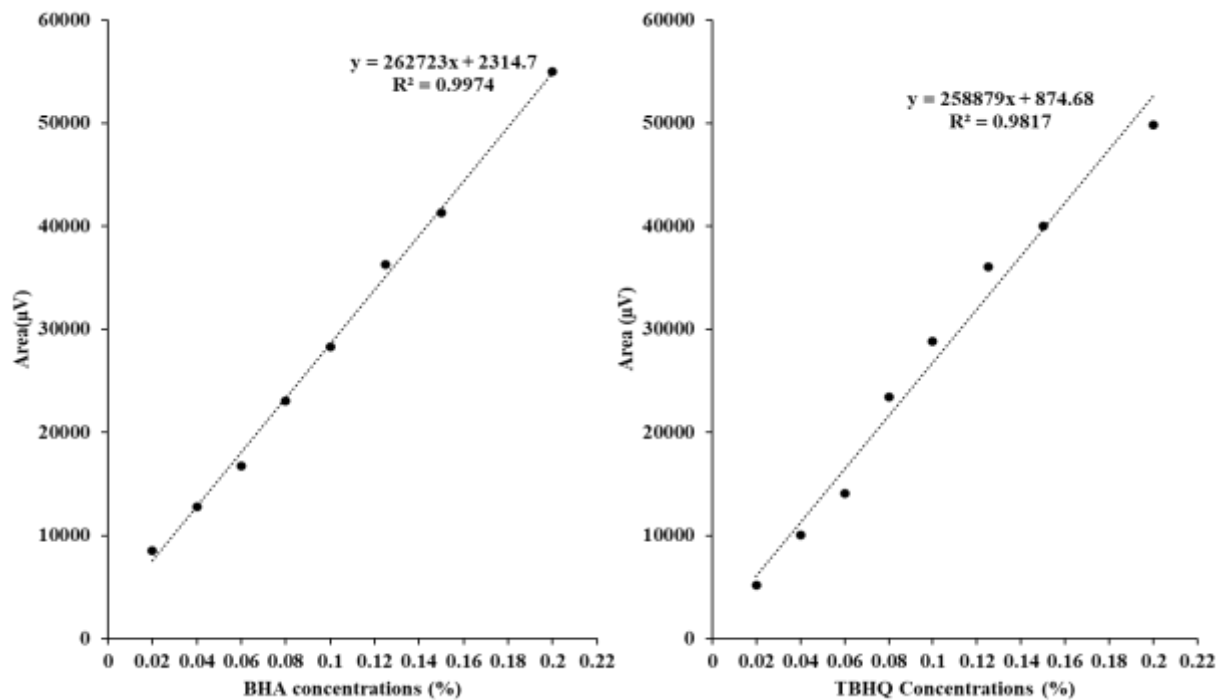


Fig 4.17. Standard curve of BHA and TBHQ in ghee without extraction (Direct method)

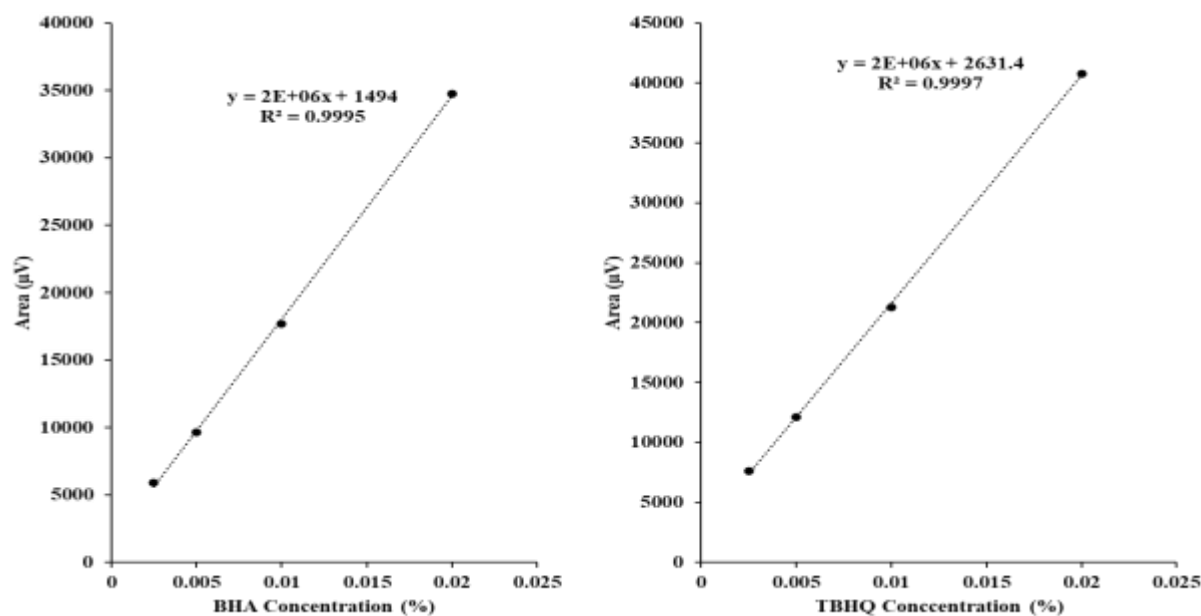


Fig 4.18 Standard curve of BHA and TBHQ in ghee (Extraction method)

4.3 Evaluation of developed rapid color based disc sensor in stored and market ghee sample

Antioxidants delay the oxidative changes in fats and oils. These compounds themselves get exhausted over a period of time as the oxidation process continues in oils/ fats. Therefore, the likelihood of affecting the sensitivity of the developed sensor was there in vegetable oil containing stored ghee samples. Therefore, the sensitivity of the developed sensor was also evaluated in stored samples and correlated with the oxidation and status of residual antioxidants in stored samples.

4.3.1 Effect of storage period on the peroxide value

Peroxide value test measures the primary oxidation products of lipid oxidation. Peroxide value expressed as mM of active oxygen/kg of ghee. Therefore, the stored samples were evaluated for their status of oxidation by peroxide value at every 15 days interval up to 9 months.

4.3.1.1 Effect of storage period on the peroxide value of pure cow ghee (PG), palm oil (PO) and PO spiked PG samples at ambient temperature (30°C)

Peroxide value was determined and expressed as mM of active oxygen/kg of ghee. These results have been depicted in Table 4.10 and Fig.4.19. It is evident from the data that peroxide value of PG remained unaltered till 60 days of storage at ambient temperature. On the contrary, in case of ghee samples spiked with PO @ 5, 7.5 and 10%, peroxide value remained unaltered till 15 days and on 30th day of analysis it showed an increase. However, the peroxide value of ghee samples spiked with PO @ 2.5% and 1.0% the increase in the peroxide value was observed at 45 and 60 days of storage, respectively. It is also evident from the data (Table 4.10) that Palm oil had the peroxide value (19.73 ± 0.27) on zero day of storage. It was also observed that in spiked ghee samples, the peroxide value significantly increased ($P < 0.05$) as the storage period progressed and observed increase was in the order of $S1 > S2 > S3 > S4$ and $S5$ (28.80 ± 0.53 , 26.80 ± 0.71 , 25.33 ± 1.33 , 23.73 ± 0.71 and 22.13 ± 0.27), respectively. This increase was corresponding to the level of added PO in the ghee. The peroxide value of control PG significantly increased ($P < 0.05$) from 0 on 0 days as the storage period progressed beyond 60 days and reached to 20.40 ± 0.71 after 9 months (270 days), which was close to the 0-day value of palm oil.

The result of ANOVA (Table 4.22) showed that the treatment i.e., palm oil spiked ghee samples had significant effect on the peroxide value. Similarly, storage days had also significant effect on the peroxide value. The interaction effect between treatment and storage days on the development of peroxide value was affected significantly at 95% level of confidence with p -value <0.001 . The development of higher peroxide value in PO and PO spiked ghee may be attributed to the presence of higher amount of poly, mono and di-unsaturated fatty acids in palm oil.

The findings of present study are in accordance with the findings of Chaudhari *et al.*, (2016). They reported that the peroxide value of cow ghee and palm oil spiked cow ghee @20% were 0.00-3.92 and 0.00-11.26) mM O₂/kg of ghee, respectively during 0-15 days of storage at 80°C.

Gosewade *et al.* (2016) also reported that the peroxide value of cow ghee and buffalo ghee increased from (0.00-2.59) and (0.00-3.19) mM O₂/kg of ghee, respectively during 0-10 days of storage at 80°C. Another study also reported that the peroxide value of cow ghee was increased from 0.00-29.65 mM O₂/kg of ghee on storage from 1- 210 days at 37±1°C and 0.00-24.89 mM O₂/kg of ghee during storage from 1-60 days at 60±1°C (Rahila, 2016).

Almeida *et al.* (2016) also reported that the peroxide value of refined palm oil was increased significantly from (00.18-58.36) meq O₂/kg of oil from 1-12 months of storage at 26-32°C.

Table 4.10 Effect of storage period on the peroxide value of cow ghee (PG), Palm oil (PO) and PO spiked PG samples at ambient temperature (30°C)

Storage Days	Peroxide Value (mM O ₂ /kg ghee)						
	PG	PO	S1	S2	S3	S4	S5
0	0.00 ^{aA}	19.73±0.27 ^{aB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
15	0.00 ^{aA}	20.27±0.53 ^{aB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
30	0.00 ^{aA}	21.27±0.27 ^{aE}	5.33±0.27 ^{bD}	4.40±0.27 ^{bC}	3.20±0.27 ^{bB}	0.00 ^{aA}	0.00 ^{aA}
45	0.00 ^{aA}	22.93±0.53 ^{bE}	6.93±0.53 ^{bcD}	5.20±0.53 ^{bC}	4.27±0.53 ^{bcBC}	3.73±0.27 ^{bB}	0.00 ^{aA}
60	0.00 ^{aA}	23.07±0.46 ^{cF}	8.53±0.46 ^{cdE}	6.40±0.53 ^{cD}	5.33±0.46 ^{cC}	4.80±0.46 ^{cBC}	3.20±0.27 ^{bB}
75	4.27±0.27 ^{bA}	24.53±0.53 ^{cE}	9.60±0.27 ^{deD}	8.00±0.53 ^{dD}	6.93±0.53 ^{dC}	5.87±0.46 ^{dBC}	4.40±0.46 ^{bAB}
90	5.87±0.46 ^{cA}	25.60±0.27 ^{cd}	10.67±0.53 ^{efC}	9.20±0.27 ^{deBC}	8.53±0.46 ^{eB}	6.93±0.53 ^{eA}	5.60±0.71 ^{cA}
105	7.47±0.53 ^{dAB}	27.73±0.46 ^{dE}	11.73±0.46 ^{fgD}	10.40±0.53 ^{efCD}	9.07±0.53 ^{efBC}	8.53±0.53 ^{fAB}	6.80±0.53 ^{cA}
120	9.07±0.46 ^{eA}	29.87±0.53 ^{eC}	12.80±0.27 ^{ghB}	11.20±0.46 ^{fgB}	10.13±0.71 ^{fgA}	9.87±0.27 ^{gA}	8.00±0.27 ^{dA}
135	10.13±0.53 ^{eA}	31.47±0.27 ^{hC}	13.87±0.53 ^{hB}	12.40±0.27 ^{ghB}	11.20±0.27 ^{gA}	10.93±0.27 ^{hA}	9.60±0.27 ^{eA}
150	11.73±0.53 ^{fA}	32.53±0.53 ^{hD}	15.07±0.46 ^{iC}	13.60±0.53 ^{hB}	12.80±0.46 ^{hAB}	12.27±0.53 ^{iA}	11.20±0.46 ^{fA}
165	12.80±0.27 ^{fgA}	34.13±0.46 ^{gD}	16.40±0.53 ^{iC}	14.80±0.27 ^{iB}	13.87±0.53 ^{hA}	13.33±0.27 ^{iA}	12.80±0.27 ^{fA}
180	13.87±0.53 ^{ghA}	35.73±0.27 ^{hD}	17.60±0.53 ^{iC}	16.00±0.71 ^{iB}	15.20±0.46 ^{iB}	14.40±0.53 ^{hA}	13.27±0.53 ^{fA}
195	14.40±0.27 ^{hiA}	37.33±0.53 ^{iE}	18.80±0.27 ^{kD}	17.20±0.46 ^{jC}	16.40±0.53 ^{iB}	15.60±0.27 ^{iB}	14.60±0.27 ^{gA}
210	15.47±0.46 ^{ijA}	38.93±0.71 ^{jE}	20.40±0.53 ^{lD}	18.40±0.53 ^{kC}	18.00±0.71 ^{jBC}	17.07±0.53 ^{mAB}	16.53±0.53 ^{hA}
225	16.53±0.53 ^{jkA}	40.53±0.53 ^{kF}	22.00±0.27 ^{lmeE}	20.00±0.27 ^{lD}	19.60±0.53 ^{kC}	18.40±0.53 ^{nB}	17.60±0.46 ^{hiAB}
240	17.60±0.71 ^{klA}	42.13±0.27 ^{lF}	24.00±0.53 ^{mnE}	21.60±0.71 ^{mD}	20.80±0.71 ^{lC}	20.27±0.53 ^{oB}	18.67±0.53 ^{ijA}
255	18.73±0.53 ^{lA}	43.73±0.53 ^{mG}	26.40±0.53 ^{noF}	23.60±0.53 ^{nE}	22.40±0.53 ^{mD}	21.60±0.27 ^{pC}	19.73±0.46 ^{jB}
270	20.40±0.71 ^{mA}	44.80±0.71 ^{nG}	28.80±0.53 ^{pF}	26.80±0.71 ^{oE}	25.33±1.33 nD	23.73±0.71 ^{qC}	22.13±0.27 ^{kB}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; PO: Palm Oil; S1: PG spiked with PO @ 10%; S2: PG spiked with PO @ 7.5%; S3: PG spiked with PO @ 5%; S4: PG spiked with PO @ 2.5%; S5: PG spiked with PO @ 1%

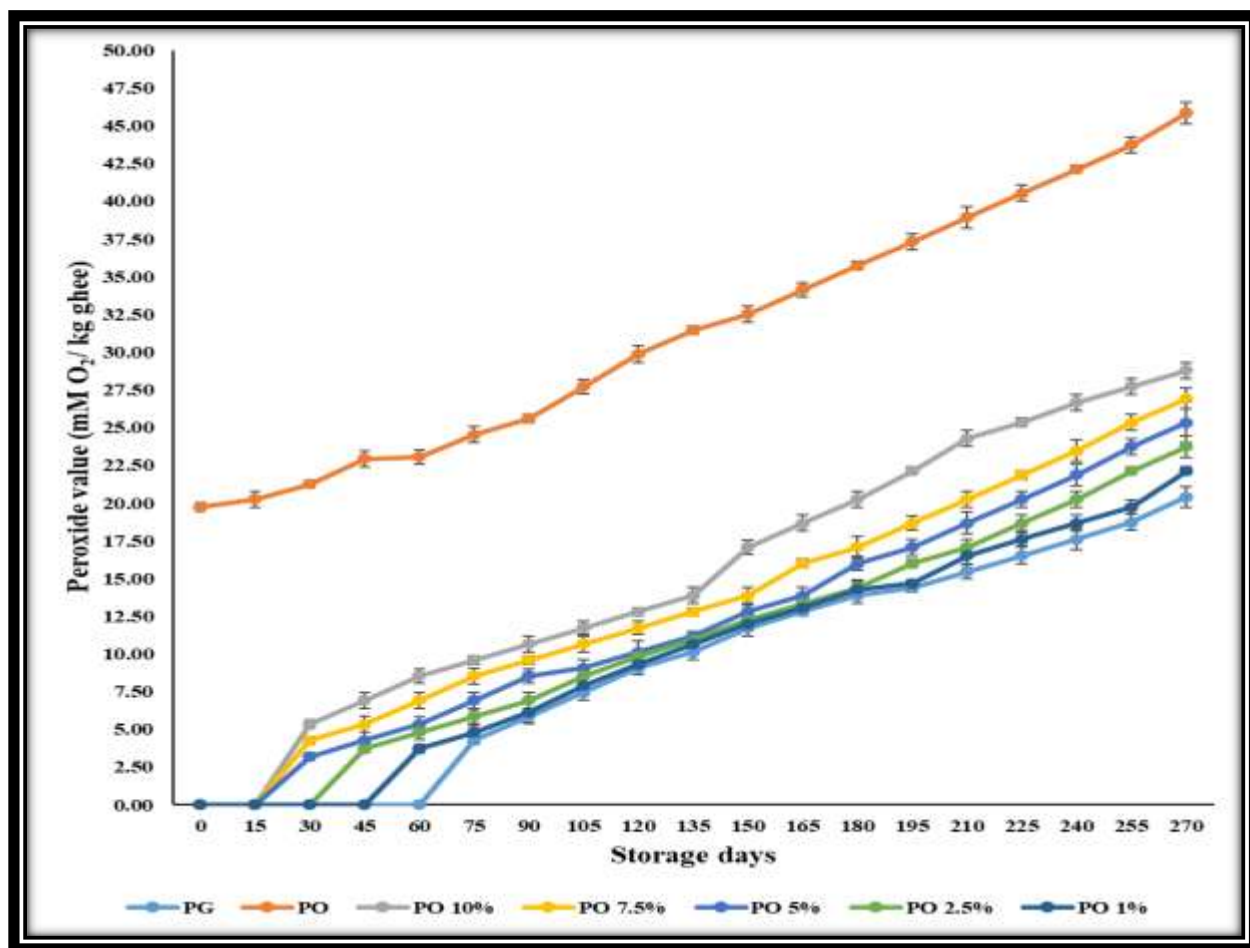


Fig. 4.19 Effect of storage period on the peroxide value of cow ghee (PG), palm oil (PO) and PO spiked PG samples at ambient temperature (30°C)

4.3.1.2 Effect of storage period on the peroxide value of PG, sunflower oil (SFO) and SFO spiked PG samples at ambient temperature (30°C)

Peroxide value was determined and expressed as mM of active oxygen/kg of ghee. These results have been depicted in Table 4.11 and Fig.4.20. It is evident from the data that peroxide value of PG remained almost nil till 60 days of storage. On the contrary, in case of ghee samples spiked with SFO @ 2.5, 5 and 7.5, 10 % showed an increase in peroxide value on 45, 30, and 30th day of storage. However, the peroxide value of ghee sample spiked with SFO @ 1% the increase in the peroxide value was observed at 60th day of storage. It is also evident from the data (Table 4.11) that SFO had the peroxide value (17.07 ± 1.07) on zero day of storage. It was also observed that in spiked ghee samples, the peroxide value significantly increased ($P < 0.05$) as the storage period progressed and increase was corresponding to the level of added SFO in the ghee. The peroxide value of control PG significantly increased ($P < 0.05$) from 0 on 0 days to 20.40 ± 0.71 after 9 months (270 days), whereas, peroxide value of SFO was increased to 41.60 ± 1.71 on 270 days of the storage. This increase was higher than those of SFO spiked PG samples in the order T1, T2, T3, T4 and T5 (26.67 ± 0.71 , 24.53 ± 0.71 , 23.20 ± 0.71 , 21.87 ± 0.53 and 20.80 ± 0.53), respectively.

The result of ANOVA (Table 4.23) illustrated that sunflower oil spiked ghee samples had significant effect on the peroxide value, similarly, storage days had also significant effect on the peroxide value. The interaction effect between treatment and storage days on the development of peroxide value was significant at 95% level of confidence with p - value < 0.001 . The higher development of peroxide value in SFO and SFO spiked ghee may be due to the higher amount of polyunsaturated fatty acids approx. (65-67%) (Aladedunye and Przybylski, 2009)

Gazwi (2017) reported that the peroxide value of sunflower oil was increased significantly from (8.50-65.20) meq O₂/kg of oil from 1-20 days of storage at 65°C.

Table 4.11 Effect of storage period on the peroxide value of PG, sunflower oil (SFO) and SFO spiked PG samples at ambient temperature (30°C)

Storage Days	Peroxide value (mM O ₂ /kg ghee)						
	PG	SFO	T1	T2	T3	T4	T5
0	0.00 ^{Aa}	17.07±1.07 ^{aB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
15	0.00 ^{aA}	17.07±1.07 ^{aB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
30	0.00 ^{aA}	18.07±1.07 ^{aC}	4.53±0.27 ^{bB}	4.00±0.27 ^{bB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
45	0.00 ^{aA}	19.20±1.00 ^{bF}	6.20±0.46 ^{cE}	5.60±0.46 ^{cB}	3.60±0.27 ^{bC}	3.20±0.27 ^{bB}	0.00 ^{aA}
60	0.00 ^{aA}	20.80±1.00 ^{bcF}	7.40±0.27 ^{cdE}	6.40±0.27 ^{dE}	4.80±0.46 ^{cd}	4.00±0.46 ^{cC}	2.93±0.27 ^{bB}
75	4.27±0.27 ^{bA}	21.87±1.53 ^{cdD}	8.80±0.46 ^{dC}	7.73±0.46 ^{deC}	6.13±0.27 ^{cB}	5.33±0.27 ^{dAB}	4.40±0.27 ^{cA}
90	5.87±0.53 ^{cA}	23.47±1.53 ^{deD}	9.40±0.27 ^{dC}	8.53±0.27 ^{eBC}	7.73±0.27 ^{dB}	7.73±0.27 ^{eB}	6.40±0.46 ^{dA}
105	7.47±0.46 ^{dA}	24.53±1.53 ^{eE}	10.93±0.27 ^{eD}	10.13±0.46 ^{fCD}	9.07±0.27 ^{eBC}	8.53±0.27 ^{eAB}	8.00±0.53 ^{eAB}
120	9.07±0.53 ^{eA}	26.67±1.53 ^{fF}	13.33±0.27 ^{fE}	12.00±0.27 ^{gDE}	10.83±0.53 ^{fCD}	10.67±0.27 ^{fBC}	9.60±0.46 ^{fAB}
135	10.13±0.53 ^{eA}	28.27±1.53 ^{fgF}	14.93±0.27 ^{gE}	13.20±0.27 ^{hDE}	12.40±0.27 ^{gCD}	12.27±0.27 ^{gBC}	11.20±0.46 ^{gAB}
150	11.73±0.46 ^{fA}	29.80±1.27 ^{gE}	15.73±0.27 ^{ghD}	14.40±0.46 ^{iD}	13.87±0.53 ^{gC}	13.07±0.27 ^{gBC}	12.27±0.27 ^{hAB}
165	12.80±0.53 ^{fgA}	30.93±1.53 ^{hF}	16.80±0.46 ^{hiE}	15.60±0.27 ^{ijD}	14.93±0.46 ^{hCD}	14.13±0.27 ^{hBC}	13.33±0.27 ^{iAB}
180	13.87±0.53 ^{ghA}	32.00±1.00 ^{hiF}	17.33±0.27 ^{ijE}	16.53±0.27 ^{jDE}	15.73±0.27 ^{hCD}	14.93±0.27 ^{hiBC}	14.20±0.46 ^{ijAB}
195	14.40±0.27 ^{hiA}	33.07±1.53 ^{ijE}	18.60±0.27 ^{jD}	17.60±0.53 ^{kCD}	16.80±0.46 ^{iC}	15.73±0.27 ^{iB}	14.93±0.27 ^{jAB}
210	15.47±0.53 ^{ijA}	34.67±1.53 ^{jkE}	20.53±0.71 ^{kD}	18.40±0.27 ^{iC}	17.87±0.27 ^{jBC}	17.07±0.27 ^{jB}	16.53±0.27 ^{kAB}
225	16.53±0.46 ^{jkA}	36.27±1.71 ^{klE}	22.29±0.56 ^{lD}	20.00±0.46 ^{mC}	18.93±0.27 ^{kBC}	18.13±0.27 ^{kB}	17.60±0.53 ^{lAB}
240	17.60±0.53 ^{klA}	37.87±1.53 ^{lmF}	23.47±0.53 ^{mE}	21.60±0.53 nD	20.53±0.46 ^{lCD}	19.47±0.27 ^{lBC}	18.67±0.46 ^{mAB}
255	18.73±0.53 ^{lA}	39.47±1.71 ^{mF}	24.80±0.46 ^{nE}	23.20±0.46 ^{oD}	21.60±0.53 ^{mC}	20.53±0.27 ^{mBC}	19.60±0.71 ^{nB}
270	20.40±0.71 ^{mA}	41.60±1.71 ^{nF}	26.67±0.71 ^{oE}	24.53±0.71 ^{pD}	23.20±0.71 ^{nC}	21.87±0.53 ^{nB}	20.80±0.53 ^{nAB}

Values are represented as Mean ± SD, n=3; a, b & A, B – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; SFO: Sunflower Oil; T1: PG spiked with SFO @ 10%; T2: PG spiked with SFO @ 7.5%; T3: PG spiked with SFO @ 5%; T4: PG spiked with SFO @ 2.5%; T5: PG spiked with SFO @ 1%

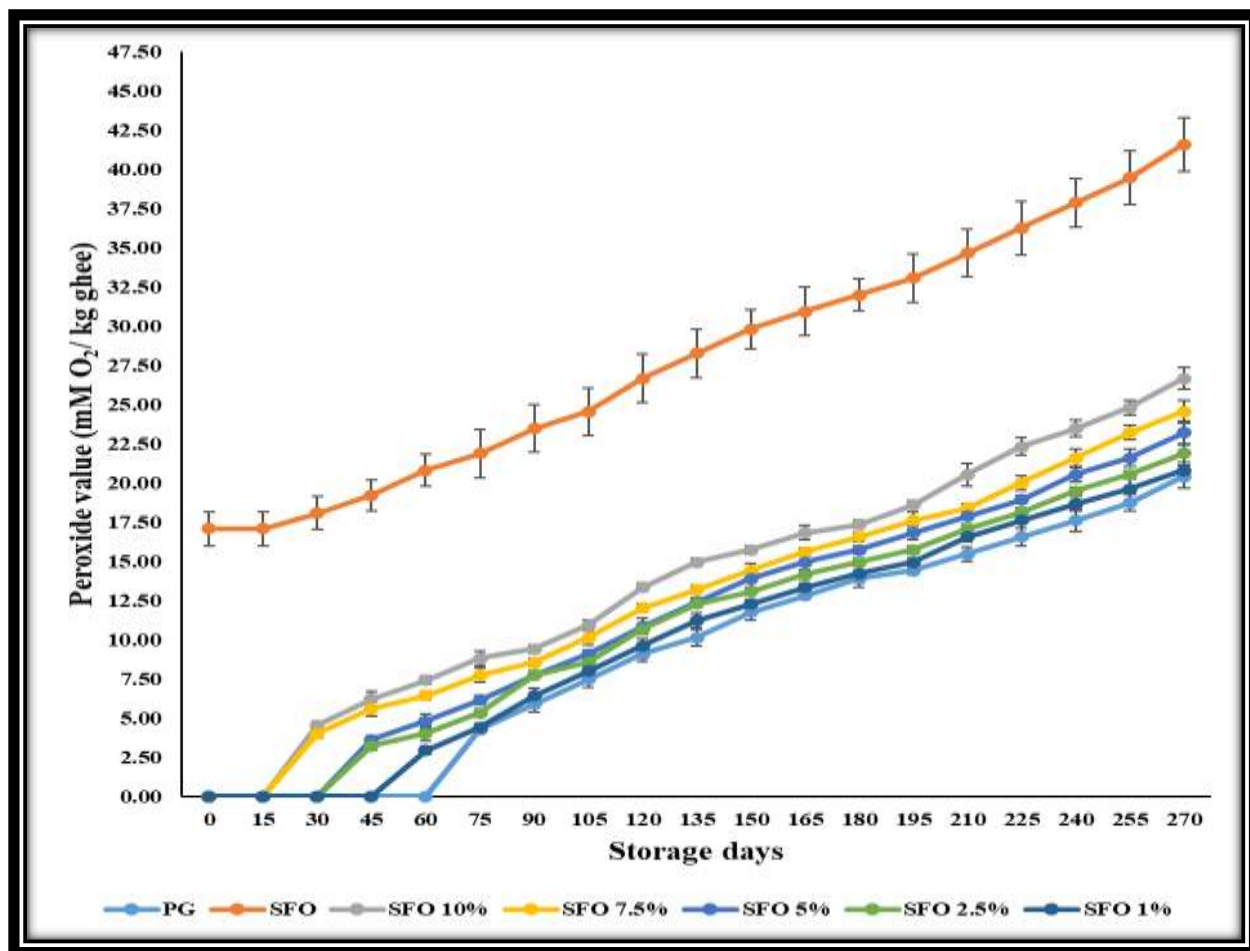


Fig 4.20 Effect of storage period on the peroxide value of PG, sunflower oil (SFO) and SFO spiked PG samples at ambient temperature (30°C)

4.3.1.3 Effect of storage period on the peroxide value of PG and TBHQ spiked PG samples at ambient temperature (30°C)

It is evident from the results (Table 4.12 and Fig 4.21) that peroxide value remained unaltered in PG samples till 60 days of storage. On the contrary, in case of ghee samples spiked with TBHQ @ 0.0025, 0.005, 0.01 and 0.02% showed an increase in peroxide value after 105, 120, 135 and 150 days, respectively. It was observed that peroxide value in ghee samples spiked with TBHQ @ 0.02% was low as compared to the samples spiked with lower concentration of TBHQ. Increase in the peroxide value on account of storage was significant ($P < 0.05$) and was inversely related to the level of added TBHQ in the ghee. The peroxide value of control PG significantly increased ($P < 0.05$) from 0 on 0 days to $20.40.73 \pm 0.71$ after 9 months (270 days).

The result of ANOVA (Table 4.24) illustrated that the treatment i.e., TBHQ spiking had significant effect on the peroxide value. Days of storage also had a significant effect on peroxide value. The interaction effect between treatment and storage days on the development of peroxide value was significant at 95% level of confidence with p -value < 0.001 . The higher development in peroxide value of PG sample may be due to the more primary oxidation products formed in lack of antioxidant. Asa et al (2015), also reported the same findings and reasoning for ghee added with BHA and orange peel extract. Rahila (2016) also reported that the peroxide value of cow ghee added with BHA (synthetic antioxidant) was increased from 0.00 on 1st day of storage at $37 \pm 1^\circ\text{C}$ to $19.69 \text{ mM O}_2/\text{kg}$ after 210 days. She also reported that the increase was from 0.00 on 1st day of storage at $60 \pm 1^\circ\text{C}$ to $23.02 \text{ mM O}_2/\text{kg}$ of ghee after 60 days.

Table 4.12 Effect of storage period on the peroxide value of PG and antioxidant (TBHQ) spiked PG samples at ambient temperature (30°C)

Storage Days	Peroxide value (mM O ₂ /kg ghee)				
	PG	U1	U2	U3	U4
0	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
15	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
30	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
45	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
60	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
75	4.27±0.27 ^{bB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
90	5.87±0.53 ^{cB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
105	7.47±0.46 ^{dB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
120	9.07±0.53 ^{eC}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	3.60±0.27 ^{bB}
135	10.13±0.53 ^{eC}	0.00 ^{aA}	0.00 ^{aA}	3.2±0.27 ^{bB}	4.80±0.27 ^{cB}
150	11.73±0.46 ^{fD}	0.00 ^{aA}	2.80±0.27 ^{bB}	4.53±0.27 ^{cBC}	5.60±0.27 ^{dC}
165	12.80±0.53 ^{fgE}	1.60±0.27 ^{bA}	3.60±0.27 ^{cB}	5.20±0.27 ^{dC}	6.80±0.27 ^{eD}
180	13.87±0.53 ^{ghD}	2.40±0.27 ^{cA}	4.40±0.27 ^{dAB}	6.00±0.27 ^{eBC}	8.00±0.27 ^{fC}
195	14.40±0.27 ^{hiE}	3.20±0.27 ^{dA}	6.40±0.27 ^{eB}	7.20±0.27 ^{fC}	9.60±0.27 ^{gD}
210	15.47±0.53 ^{ijD}	4.00±0.27 ^{eA}	7.60±0.27 ^{fAB}	8.00±0.27 ^{gBC}	11.20±0.27 ^{hC}
225	16.53±0.46 ^{jkD}	4.80±0.27 ^{fA}	8.40±0.46 ^{gAB}	9.20±0.27 ^{hB}	12.80±0.46 ^{iC}
240	17.60±0.53 ^{klE}	6.00±0.27 ^{gA}	9.60±0.27 ^{hB}	10.40±0.46 ^{iC}	14.40±0.53 ^{jD}
255	18.73±0.53 ^{lD}	7.60±0.46 ^{hA}	11.2±0.46 ^{iB}	12.00±0.53 ^{jC}	16.40±0.71 ^{kC}
270	20.40±0.71 ^{mD}	9.60±0.46 ^{iA}	12.40±0.53 ^{jB}	14.00±0.71 ^{kB}	18.40±0.71 ^{lC}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and Column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; U1: PG spiked with TBHQ @ 0.02%; U2: PG spiked with TBHQ @ 0.01%; U3: PG spiked with TBHQ @ 0.005%; U4: PG spiked with TBHQ @ 0.0025%

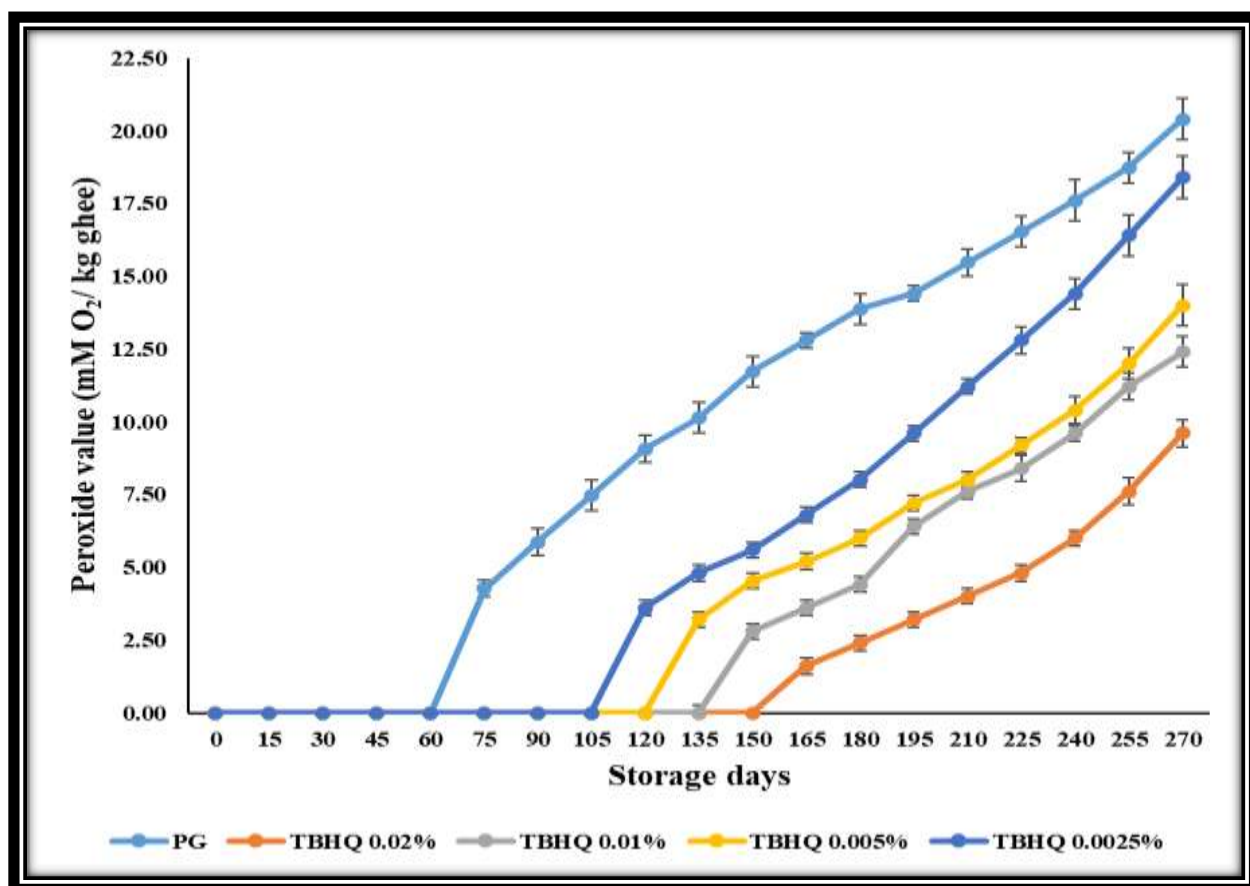


Fig 4.21 Effect of storage period on the peroxide value of PG and TBHQ spiked PG samples at ambient temperature (30°C)

4.3.1.4 Effect of storage period on the peroxide value of PG and BHA spiked PG samples at ambient temperature (30°C)

These results have been depicted in Table 4.13 and Fig.4.22. It is evident from the data that peroxide value of PG sample as well as ghee sample containing lowest level of BHA i.e., V4 remained unaltered till 60 days of storage at ambient temperature. On the contrary, in case of ghee samples spiked with BHA @ 0.005, 0.01 and 0.02%, the increase in peroxide value was observed after 75, 90 and 105 days, respectively. It is also evident from the results that on account of storage there was increase in the peroxide value of all types of the samples but this increase was lowest and slowest in case of ghee samples containing BHA @ 0.02% and increase was corresponding to the added BHA in the ghee. The peroxide value of control PG significantly increased ($P < 0.05$) from 0 on 0 days to $20.40.73 \pm 0.71$ after 9 months (270 days).

Statistical analysis (Table 4.25) illustrated that the treatment i.e., BHA spiked ghee samples had significant effect on the peroxide value similarly, the storage days had also significant effect on peroxide value. The interaction effect between treatment and storage days on the development of peroxide value was significant at 95% level of confidence with p -value < 0.001 . The higher development of peroxide value in PG sample may be due to the more primary oxidation products formed in lack of antioxidant. Similar results were reported when ghee was treated with BHA and orange peel extract (Asa et al., 2015 and Rahila, 2016).

Table 4.13 Effect of storage period on the peroxide value of PG and BHA spiked PG samples at ambient temperature (30°C)

Storage Days	Peroxide value (mM O ₂ /kg ghee)				
	PG	V1	V2	V3	V4
0	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
15	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
30	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
45	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
60	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}
75	4.27±0.27 ^{bB}	0.00 ^{aA}	0.00 ^{aA}	0.00 ^{aA}	4.00±0.46 ^{bB}
90	5.87±0.53 ^{cC}	0.00 ^{aA}	0.00 ^{aA}	3.60±0.46 ^{bB}	5.33±0.27 ^{cB}
105	7.47±0.46 ^{dD}	0.00 ^{aA}	3.20±0.46 ^{bB}	4.80±0.27 ^{cBC}	6.93±0.27 ^{dC}
120	9.07±0.53 ^{eD}	2.00±0.46 ^{bA}	4.00±0.27 ^{bAB}	5.60±0.27 ^{dBC}	8.00±0.27 ^{eC}
135	10.13±0.53 ^{eD}	2.80±0.27 ^{bA}	4.80±0.27 ^{cAB}	6.80±0.27 ^{eBC}	9.20±0.27 ^{fC}
150	11.73±0.46 ^{fD}	3.60±0.27 ^{cA}	6.00±0.27 ^{dAB}	8.00±0.27 ^{fB}	10.40±0.00 ^{gC}
165	12.80±0.53 ^{fgC}	4.40±0.27 ^{dA}	7.20±0.27 ^{eA}	9.20±0.00 ^{gB}	11.47±0.46 ^{hB}
180	13.87±0.53 ^{ghD}	5.60±0.27 ^{eA}	8.40±0.00 ^{fAB}	10.40±0.46 ^{hB}	12.27±0.46 ^{iC}
195	14.40±0.27 ^{hiD}	6.80±0.00 ^{fA}	9.60±0.46 ^{gA}	11.60±0.46 ^{iB}	13.87±0.27 ^{jC}
210	15.47±0.53 ^{ijD}	8.00±0.46 ^{gA}	10.80±0.46 ^{hB}	12.80±0.27 ^{jBC}	14.67±0.27 ^{kC}
225	16.53±0.46 ^{klD}	9.60±0.46 ^{hA}	12.00±0.27 ^{iAB}	14.00±0.27 ^{kBC}	15.47±0.27 ^{lC}
240	17.60±0.53 ^{klE}	11.20±0.27 ^{iA}	13.60±0.27 ^{jB}	15.60±0.27 ^{lC}	16.53±0.27 ^{mD}
255	18.73±0.53 ^{lD}	12.80±0.27 ^{jA}	15.20±0.27 ^{kAB}	16.80±0.27 ^{mBC}	17.60±0.27 ^{nC}
270	20.40±0.71 ^{mD}	14.40±0.27 ^{KA}	16.80±0.27 ^{LAB}	18.40±0.27 ^{nBC}	19.20±0.27 ^{nC}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; V1: PG spiked with BHA @ 0.02%; V2: PG spiked with BHA @ 0.01%; V3: PG spiked with BHA @ 0.005%; V4: PG spiked with BHA @ 0.0025%

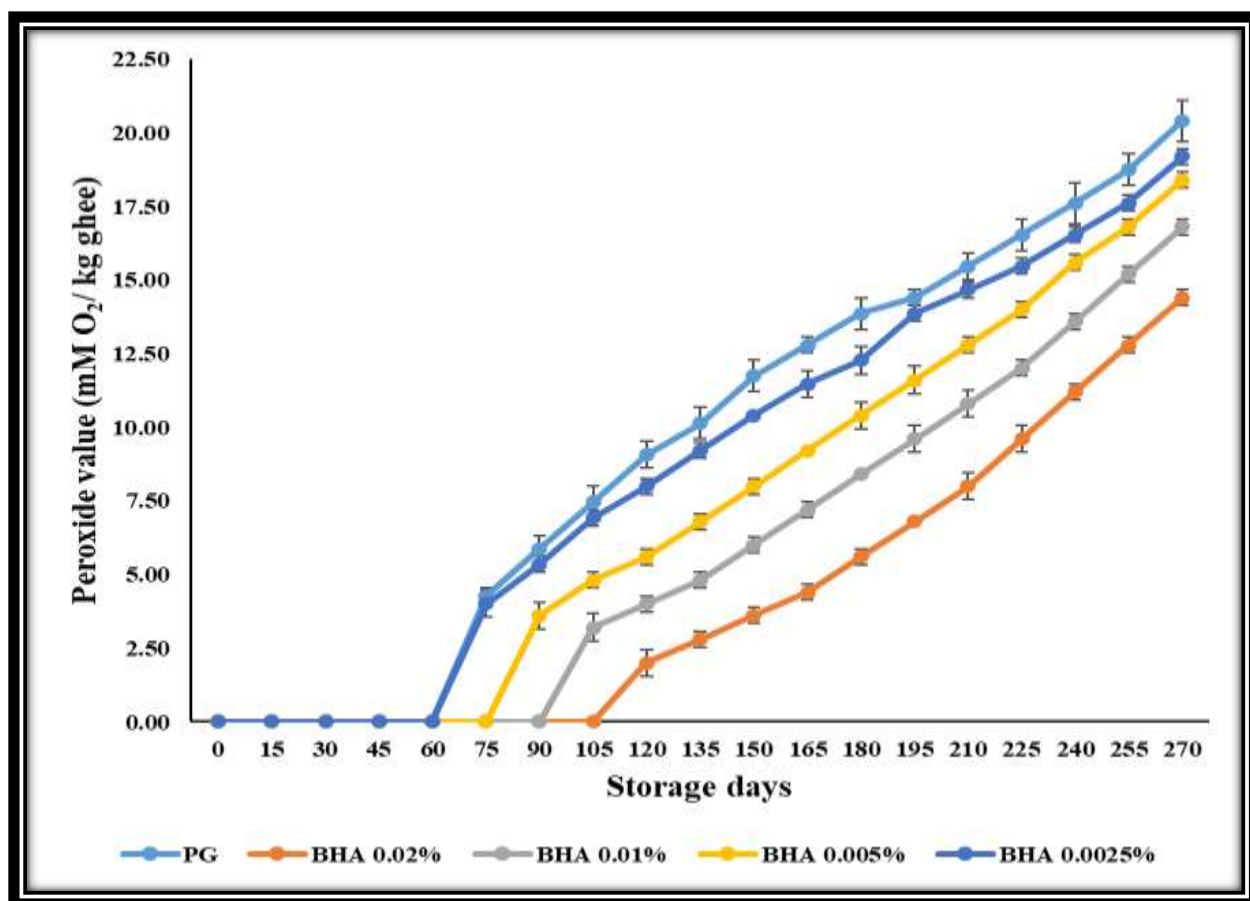


Fig 4.22 Effect of storage period on the peroxide value of PG and BHA spiked PG samples at ambient temperature (30°C)

4.3.2 Effect of storage period on the TBA-value

TBA value test measures the secondary oxidation products of lipid oxidation. TBA value expressed as malondialdehyde (MDA) (mg %) in 0.1 g of fat. Therefore, the stored samples were evaluated for their status of oxidation by TBA- value at every 15 days interval up to 9 months.

4.3.2.1 Effect of storage period on the TBA-value of PG, PO and PO spiked PG samples at ambient temperature (30°C)

TBA value was determined and expressed as TBARS/0.1g of ghee. These results have been depicted in Table 4.14 and Fig 4.23. It is evident from the data that PG as well as PO and PO spiked with PG samples showed an increase in the TBA value from the 0 days onwards. It is also evident from the data (Table 4.14) that PO had the TBA value (0.007 ± 0.001) on zero day of storage. It was also observed that in spiked ghee samples, the TBA value significantly increased ($P < 0.05$) as the storage period progressed and increase was corresponding to the level of added PO in the ghee. The TBA value of control PG significantly increased ($P < 0.05$) from 0.019 ± 0.001 on 0 days to 0.073 ± 0.004 after 9 months (270 days), whereas, TBA value of PO was increased to 0.040 ± 0.004 on 270 days of the storage. This increase was lower than those of PO spiked PG samples in the order Sa, Sb, Sc, Sd and Se (0.061 ± 0.003 , 0.064 ± 0.002 , 0.066 ± 0.002 , 0.068 ± 0.001 and 0.070 ± 0.003), respectively

The result in Table 4.22 indicated that the treatment i.e., palm oil spiked ghee samples had significant effect on the TBA value, similarly storage days had also significant effect on the TBA value. The interaction effect between treatment and storage days on the development of TBA was significant at 95% level of confidence with p - value < 0.001 . Comparatively high TBA value of ghee may be attributed to the fact that pure ghee did not contain any added synthetic antioxidant, whereas PO and PO spiked ghee samples had the prevention of oxidation due to the antioxidant present in PO. The SFA is of PO and PG is close and varied in the PO (48-50%), whereas in case of PG (65-66%). The PUFA of both PO and PG is also close and varied from (10-11%) and (4-5%), respectively (Montoya *et al.*, 2014; Pena-Serna and Restrepo-Betancur, 2020).

Table 4.14 Effect of storage period on the TBA-value of PG, PO and PO spiked PG samples at ambient temperature (30°C)

Storage Days	TBA-value (TBARS/0.1g ghee)						
	PG	PO	Sa	Sb	Sc	Sd	Se
0	0.019±0.001 ^{Ab}	0.007±0.001 ^{aA}	0.015±0.001 ^{aB}	0.016±0.001 ^{aBC}	0.017±0.001 ^{aCD}	0.018±0.001 ^{aD}	0.018±0.002 ^{aCD}
15	0.020±0.002 ^{bCD}	0.008±0.000 ^{bA}	0.016±0.000 ^{abB}	0.017±0.002 ^{abBC}	0.018±0.000 ^{abD}	0.019±0.002 ^{abD}	0.019±0.001 ^{abD}
30	0.021±0.001 ^{cE}	0.009±0.001 ^{abcA}	0.017±0.001 ^{bcB}	0.018±0.002 ^{bcC}	0.019±0.000 ^{bcCD}	0.020±0.000 ^{bDE}	0.020±0.001 ^{bDE}
45	0.022±0.001 ^{cdE}	0.010±0.001 ^{bcdA}	0.018±0.002 ^{cdB}	0.019±0.001 ^{cdBC}	0.020±0.002 ^{cdCD}	0.021±0.001 ^{bDE}	0.021±0.001 ^{bcDE}
60	0.023±0.001 ^{dD}	0.011±0.001 ^{cdA}	0.019±0.002 ^{deB}	0.020±0.001 ^{cdB}	0.021±0.001 ^{deC}	0.022±0.000 ^{bC}	0.023±0.001 ^{cdCD}
75	0.024±0.001 ^{dE}	0.012±0.002 ^{deA}	0.020±0.002 ^{efB}	0.021±0.000 ^{deBC}	0.022±0.001 ^{eCD}	0.023±0.002 ^{cdE}	0.024±0.001 ^{deDE}
90	0.026±0.003 ^{eD}	0.014±0.000 ^{efA}	0.022±0.001 ^{fB}	0.022±0.002 ^{eB}	0.024±0.001 ^{fC}	0.025±0.001 ^{dC}	0.025±0.001 ^{eC}
105	0.028±0.002 ^{fE}	0.016±0.002 ^{fgA}	0.023±0.001 ^{gB}	0.025±0.002 ^{fBC}	0.026±0.001 ^{gCD}	0.027±0.001 ^{deCD}	0.027±0.002 ^{fD}
120	0.031±0.002 ^{gF}	0.018±0.001 ^{gA}	0.025±0.001 ^{hB}	0.026±0.000 ^{fBC}	0.027±0.000 ^{gCD}	0.028±0.001 ^{eDE}	0.029±0.001 ^{fgE}
135	0.033±0.002 ^{gE}	0.020±0.003 ^{hA}	0.027±0.002 ^{iB}	0.028±0.001 ^{gB}	0.029±0.001 ^{hC}	0.030±0.001 ^{fCD}	0.031±0.000 ^{gD}
150	0.035±0.001 ^{hF}	0.022±0.002 ^{iA}	0.029±0.001 ^{jB}	0.030±0.001 ^{hBC}	0.031±0.003 ^{icCD}	0.032±0.001 ^{gD}	0.033±0.002 ^{hE}
165	0.037±0.001 ^{iE}	0.024±0.002 ^{ijA}	0.031±0.002 ^{kB}	0.032±0.002 ^{hB}	0.033±0.001 ^{jC}	0.034±0.001 ^{hCD}	0.035±0.003 ^{hD}
180	0.039±0.001 ^{jF}	0.026±0.001 ^{jkA}	0.033±0.001 ^{iB}	0.034±0.001 ^{iBC}	0.035±0.001 ^{kCD}	0.036±0.001 ^{iDE}	0.037±0.002 ^{iE}
195	0.042±0.002 ^{kD}	0.028±0.003 ^{klA}	0.035±0.002 ^{mB}	0.037±0.001 ^{jBC}	0.039±0.001 ^{lC}	0.039±0.001 ^{jC}	0.040±0.003 ^{jC}
210	0.046±0.003 ^{lE}	0.030±0.002 ^{lA}	0.038±0.002 ^{nB}	0.040±0.002 ^{kC}	0.042±0.001 ^{mD}	0.043±0.001 ^{kD}	0.044±0.001 ^{kD}
225	0.052±0.002 ^{mF}	0.032±0.003 ^{mA}	0.041±0.001 ^{oB}	0.044±0.002 ^{lC}	0.046±0.001 ^{nCD}	0.048±0.002 ^{iDE}	0.050±0.002 ^{lE}
240	0.058±0.002 ^{nF}	0.034±0.001 ^{nA}	0.045±0.001 ^{pB}	0.049±0.001 ^{mC}	0.052±0.003 ^{oD}	0.054±0.001 ^{mE}	0.056±0.001 ^{mE}
255	0.065±0.003 ^{oF}	0.037±0.002 ^{oA}	0.052±0.002 ^{qB}	0.056±0.002 ^{nC}	0.058±0.002 ^{pCD}	0.060±0.001 ^{nDE}	0.062±0.002 ^{nE}
270	0.073±0.004 ^{pF}	0.040±0.004 ^{pA}	0.061±0.003 ^{rB}	0.064±0.002 ^{oBC}	0.066±0.002 ^{qCD}	0.068±0.001 ^{oDE}	0.070±0.003 ^{oE}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; PO: Palm Oil; Sa: PG spiked with PO @ 10%; Sb: PG spiked with PO @ 7.5%; Sc: PG spiked with PO @ 5%; Sd: PG spiked with PO @ 2.5%; Se: PG spiked with PO @ 1%;

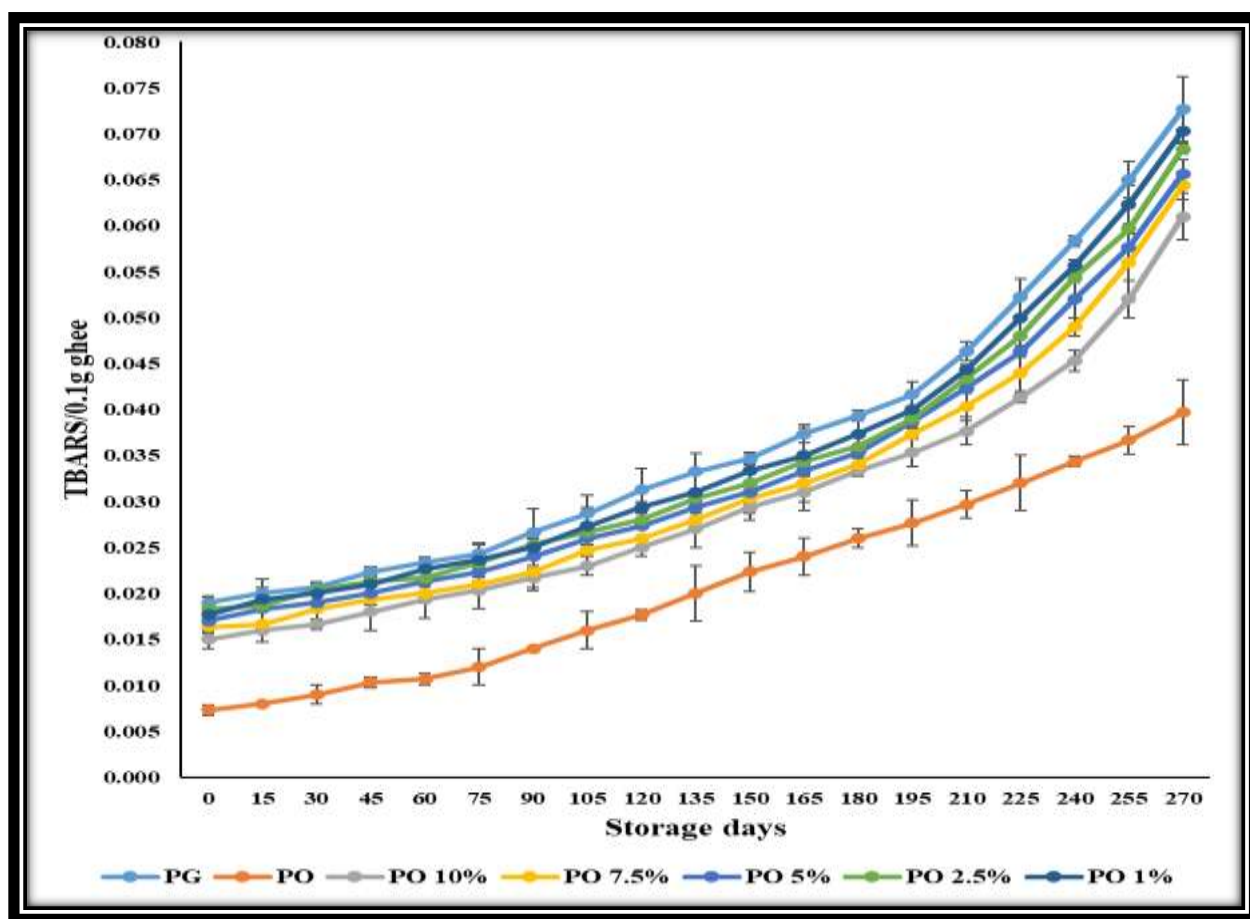


Fig 4.23 Effect of storage period on the TBA-value of PG, PO and PO spiked PG samples at ambient temperature (30°C)

4.3.2.2 Effect of storage period on the TBA-value of PG, SFO and SFO spiked PG samples at ambient temperature (30°C)

It is evident from the data (Table 4.15 and Fig 4.24) that PG as well as SFO and SFO spiked PG samples showed an increase in the TBA value on account of storage. It is also evident from the data (Table 4.15) that sunflower oil had the TBA value (0.099 ± 0.001) on 0 day of storage. It was also observed that in both SFO and spiked ghee samples, the TBA value significantly increased ($P < 0.05$) as the storage period progressed and increase was corresponding to the level of added SFO in the ghee. The TBA value of control PG significantly increased ($P < 0.05$) from 0.019 ± 0.001 on 0 days to 0.073 ± 0.004 after 9 months (270 days), whereas, TBA value of SFO was increased to 0.281 ± 0.006 on 270 days of the storage vis'-a-vis' 0.099 ± 0.001 on 0 day. This increase was higher than those of SFO spiked PG samples in the order $T_a > T_b > T_c > T_d > T_e$ (0.094 ± 0.001 , 0.088 ± 0.001 , 0.084 ± 0.001 , 0.082 ± 0.001 , 0.081 ± 0.001), respectively.

The result in Table 4.23 indicated that the treatment i.e. SFO spiked ghee samples had significant effect on the peroxide value, similarly storage days had also significant effect on the peroxide value. The interaction effect between treatment and storage days on the development of TBA was significant at 95% level of confidence with p -value < 0.001 . The higher development of TBA value in SFO and SFO spiked ghee samples (S_a , S_b , S_c , S_d and S_e) than PG. The reason behind that the approx. 65-67% poly unsaturated fatty acid available in SFO.

Gazwi (2017) reported that the TBA value of sunflower oil was increased significantly from (1.144-12.65.20) mg malondialdehyde/kg of oil from 1-20 days of storage at 65°C.

Table 4.15 Effect of storage period on the TBA value of PG, SFO and SFO spiked PG samples at ambient temperature (30°C)

Storage Days	TBA-value (TBARS/0.1g ghee)						
	PG	SFO	Ta	Tb	Tc	Td	Te
0	0.019±0.001 ^{aA}	0.099±0.001 ^{aD}	0.022±0.001 ^{aC}	0.021±0.000 ^{aBC}	0.020±0.001 ^{aABC}	0.019±0.000 ^{aABC}	0.019±0.001 ^{aAB}
15	0.020±0.002 ^{bA}	0.104±0.001 ^{bD}	0.023±0.001 ^{bC}	0.022±0.001 ^{bBC}	0.021±0.001 ^{bABC}	0.020±0.002 ^{bAB}	0.020±0.001 ^{bAB}
30	0.021±0.001 ^{cA}	0.115±0.002 ^{cE}	0.026±0.001 ^{cD}	0.025±0.001 ^{cCD}	0.024±0.002 ^{cBC}	0.023±0.001 ^{cB}	0.022±0.001 ^{cAB}
45	0.022±0.001 ^{cdA}	0.123±0.001 ^{dE}	0.028±0.001 ^{cdD}	0.027±0.000 ^{cdCD}	0.025±0.002 ^{cdBC}	0.024±0.001 ^{cdAB}	0.023±0.002 ^{cA}
60	0.023±0.001 ^{dA}	0.127±0.001 ^{dD}	0.030±0.001 ^{deC}	0.029±0.002 ^{deC}	0.028±0.001 ^{dB}	0.025±0.001 ^{dAB}	0.024±0.003 ^{cdA}
75	0.024±0.001 ^{dA}	0.133±0.001 ^{eE}	0.032±0.001 ^{eD}	0.031±0.001 ^{eD}	0.029±0.001 ^{eC}	0.027±0.001 ^{eB}	0.026±0.001 ^{dA}
90	0.026±0.003 ^{eA}	0.145±0.001 ^{fF}	0.035±0.002 ^{fE}	0.034±0.001 ^{fDE}	0.032±0.001 ^{fCD}	0.031±0.000 ^{fBC}	0.029±0.001 ^{efAB}
105	0.028±0.002 ^{fA}	0.154±0.001 ^{gF}	0.037±0.001 ^{gE}	0.036±0.002 ^{fgDE}	0.035±0.001 ^{fgCD}	0.033±0.001 ^{gBC}	0.032±0.002 ^{gAB}
120	0.031±0.002 ^{gA}	0.163±0.001 ^{hE}	0.040±0.002 ^{hD}	0.038±0.001 ^{gC}	0.037±0.001 ^{gBC}	0.035±0.002 ^{gAB}	0.034±0.002 ^{gAB}
135	0.033±0.002 ^{gA}	0.175±0.005 ^{iE}	0.043±0.001 ^{hD}	0.041±0.001 ^{hCD}	0.039±0.001 ^{hBCD}	0.036±0.001 ^{hABC}	0.035±0.001 ^{hAB}
150	0.035±0.001 ^{hA}	0.184±0.002 ^{jE}	0.046±0.001 ^{iD}	0.043±0.003 ^{hC}	0.041±0.002 ^{iBC}	0.040±0.001 ^{hAB}	0.038±0.001 ^{iAB}
165	0.037±0.001 ^{iA}	0.199±0.002 ^{keE}	0.050±0.002 ^{jD}	0.045±0.001 ^{iC}	0.044±0.001 ^{jBC}	0.042±0.003 ^{iAB}	0.040±0.003 ^{iA}
180	0.039±0.001 ^{jAB}	0.202±0.001 ^{kF}	0.055±0.001 ^{keE}	0.051±0.002 ^{jD}	0.047±0.001 ^{kCD}	0.045±0.001 ^{jBC}	0.041±0.003 ^{jA}
195	0.042±0.002 ^{kA}	0.209±0.002 ^{lE}	0.060±0.002 ^{lD}	0.053±0.001 ^{kC}	0.051±0.001 ^{lC}	0.049±0.001 ^{kB}	0.048±0.001 ^{kAB}
210	0.046±0.003 ^{lA}	0.222±0.002 ^{mF}	0.066±0.001 ^{mE}	0.061±0.001 ^{lD}	0.059±0.001 ^{mD}	0.057±0.001 ^{lC}	0.054±0.001 ^{lB}
225	0.052±0.002 ^{mA}	0.239±0.003 ^{nF}	0.072±0.001 ^{nE}	0.067±0.001 ^{mD}	0.065±0.001 ^{nCD}	0.064±0.001 ^{mBC}	0.062±0.001 ^{mB}
240	0.058±0.002 ^{nA}	0.257±0.004 ^{oE}	0.080±0.002 ^{oD}	0.075±0.001 ^{nC}	0.073±0.001 ^{oBC}	0.070±0.001 ^{nAB}	0.068±0.001 ^{nA}
255	0.065±0.003 ^{oA}	0.272±0.004 ^{pE}	0.087±0.002 ^{pD}	0.082±0.002 ^{oC}	0.079±0.002 ^{pBC}	0.077±0.002 ^{oAB}	0.076±0.001 ^{oA}
270	0.073±0.004 ^{pA}	0.281±0.006 ^{qE}	0.094±0.001 ^{qD}	0.088±0.001 ^{pC}	0.084±0.001 ^{qBC}	0.082±0.001 ^{pAB}	0.081±0.001 ^{pAB}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; SFO: Sunflower Oil; Ta: PG spiked with SFO @ 10%; Tb: PG spiked with SFO @ 7.5%; Tc: PG spiked with SFO @ 5%; Td: PG spiked with SFO @ 2.5%; Te: PG spiked with SFO @ 1%;

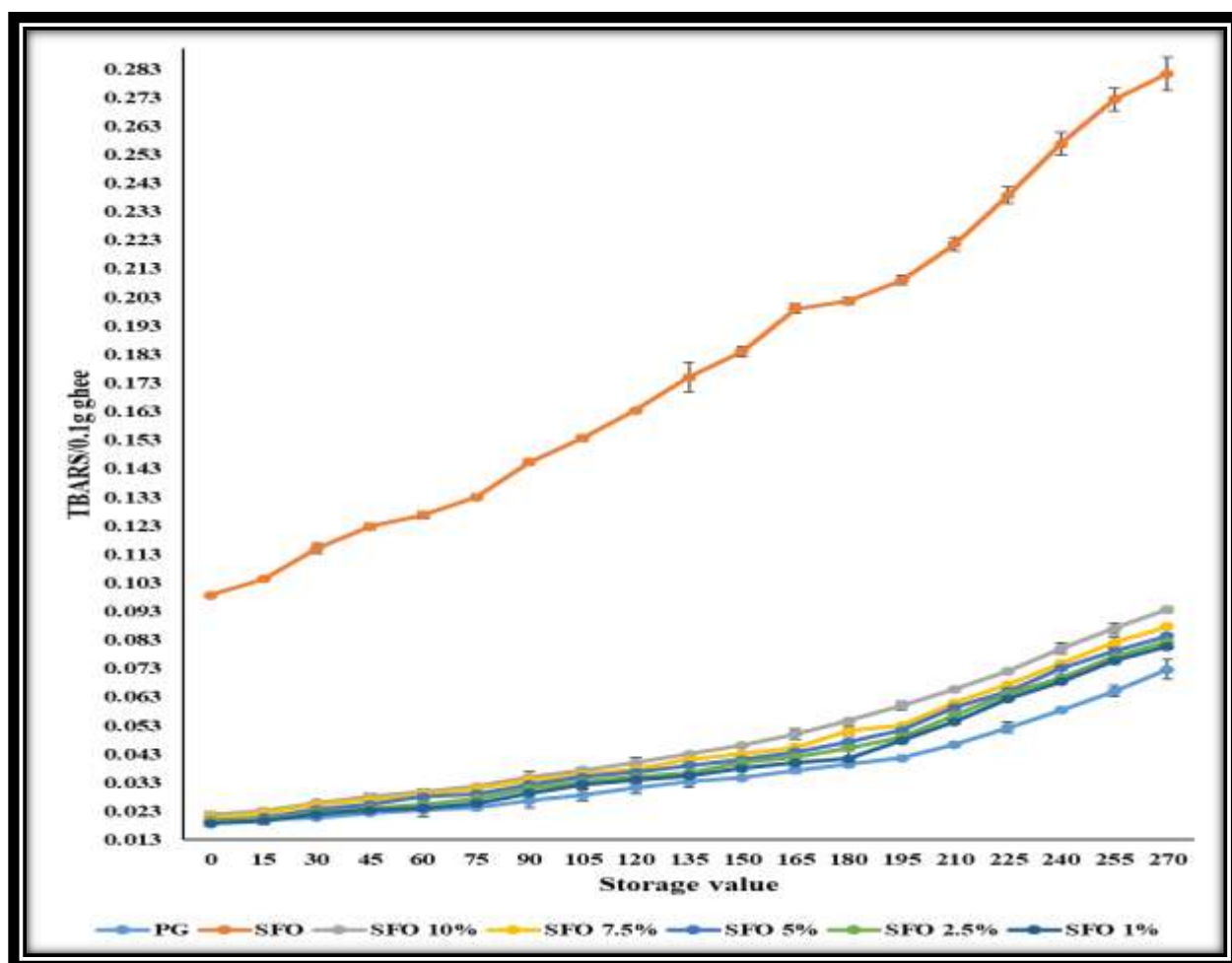


Fig 4.24 Effect of storage period on the TBA value of PG, SFO and SFO spiked PG samples at ambient temperature (30°C)

4.3.2.3 Effect of storage period on the TBA-value of PG and TBHQ spiked PG samples at ambient temperature (30°C)

It is evident from the data (Table 4.16 and Fig.4.25) that PG as well as TBHQ spiked ghee samples showed an increase in the TBA value from the 0 days onwards. It was observed that in TBHQ spiked ghee samples, the TBA value significantly increased ($P < 0.05$) as the storage period progressed and increase was corresponding to the added TBHQ in the ghee. The TBA value of control PG significantly increased ($P < 0.05$) from 0.019 ± 0.001 on 0 days to 0.073 ± 0.004 after 9 months (270 days). On the contrary, in case of TBHQ spiked ghee samples, this increase was lower than the ghee containing no added TBHQ. Even in the lowest added concentration of TBHQ i.e. 0.0025%, the TBA value measured after 270 days of storage was 0.041 ± 0.001 , which was just the half of the value reported in PG samples without any TBHQ. This clearly indicated that the addition of TBHQ helped in controlling the rate of oxidation of ghee during storage.

The result of ANOVA (Table 4.24) illustrated that the treatment i.e., TBHQ spiked ghee samples had significant effect on the TBA value similarly, the storage days had also significant effect on TBA value. The interaction effect between treatment and storage days on the development of TBA was significant at 95% level of confidence with p -value < 0.001 . The PG sample highest increase in TBA value may be due to the more secondary oxidation products formed during storage.

Table 4.16 Effect of storage period on the TBA-value of PG and TBHQ spiked PG samples at ambient temperature (30°C)

Storage Days	TBA-value (TBARS/0.1g ghee)				
	PG Pure	Ua	Ub	Uc	Ud
0	0.019±0.001 ^{aC}	0.006±0.001 ^{aA}	0.007±0.001 ^{aA}	0.009±0.001 ^{aB}	0.014±0.001 ^{aC}
15	0.020±0.002 ^{bD}	0.007±0.001 ^{aA}	0.008±0.000 ^{bcA}	0.012±0.002 ^{abB}	0.016±0.001 ^{aC}
30	0.021±0.001 ^{cD}	0.007±0.002 ^{aA}	0.009±0.001 ^{cA}	0.013±0.001 ^{bB}	0.017±0.001 ^{bC}
45	0.022±0.001 ^{cdC}	0.009±0.002 ^{abA}	0.010±0.001 ^{dA}	0.015±0.001 ^{cB}	0.019±0.001 ^{bcB}
60	0.023±0.001 ^{dD}	0.011±0.001 ^{bcA}	0.012±0.001 ^{eB}	0.016±0.001 ^{cdC}	0.020±0.001 ^{cC}
75	0.024±0.001 ^{dD}	0.011±0.001 ^{bcA}	0.014±0.001 ^{fB}	0.018±0.001 ^{dc}	0.021±0.002 ^{dC}
90	0.026±0.003 ^{eD}	0.013±0.001 ^{cdA}	0.017±0.001 ^{fgB}	0.020±0.001 ^{deBC}	0.022±0.001 ^{dC}
105	0.028±0.002 ^{fE}	0.014±0.001 ^{deA}	0.019±0.001 ^{gB}	0.021±0.002 ^{eC}	0.025±0.001 ^{eD}
120	0.031±0.002 ^{gD}	0.016±0.003 ^{efA}	0.021±0.001 ^{hB}	0.025±0.001 ^{fC}	0.028±0.003 ^{fC}
135	0.033±0.002 ^{gD}	0.018±0.003 ^{fgA}	0.022±0.001 ^{iB}	0.027±0.003 ^{gC}	0.030±0.003 ^{gC}
150	0.035±0.001 ^{hD}	0.019±0.005 ^{ghA}	0.024±0.002 ^{ijB}	0.029±0.003 ^{ghBC}	0.031±0.003 ^{ghC}
165	0.037±0.001 ^{iD}	0.022±0.003 ^{hiA}	0.026±0.002 ^{jkB}	0.031±0.003 ^{hiC}	0.032±0.002 ^{hiC}
180	0.039±0.001 ^{jE}	0.023±0.002 ^{hijA}	0.027±0.002 ^{kIB}	0.031±0.001 ^{hiC}	0.033±0.001 ^{hiD}
195	0.042±0.002 ^{KE}	0.025±0.002 ^{ijA}	0.028±0.001 ^{lmB}	0.032±0.001 ^{ijC}	0.034±0.002 ^{ijD}
210	0.046±0.003 ^{IE}	0.025±0.002 ^{jkA}	0.028±0.002 ^{mb}	0.032±0.001 ^{ijC}	0.035±0.002 ^{ijD}
225	0.052±0.002 ^{mE}	0.026±0.001 ^{jkA}	0.029±0.001 ^{mnB}	0.033±0.003 ^{ijC}	0.036±0.001 ^{jkD}
240	0.058±0.002 ^{nE}	0.027±0.001 ^{klA}	0.030±0.001 ^{nB}	0.034±0.003 ^{jC}	0.037±0.001 ^{klD}
255	0.065±0.003 ^{oD}	0.029±0.001 ^{lA}	0.032±0.001 ^{nA}	0.035±0.001 ^{jB}	0.038±0.001 ^{lC}
270	0.073±0.004 ^{pe}	0.030±0.002 ^{lA}	0.034±0.001 ^{oB}	0.036±0.002 ^{kC}	0.041±0.001 ^{md}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; Ua: PG spiked with TBHQ @ 0.02%; Ub: PG spiked with TBHQ @ 0.01%; Uc: PG spiked with TBHQ @ 0.005%; Ud: PG spiked with TBHQ @ 0.0025%

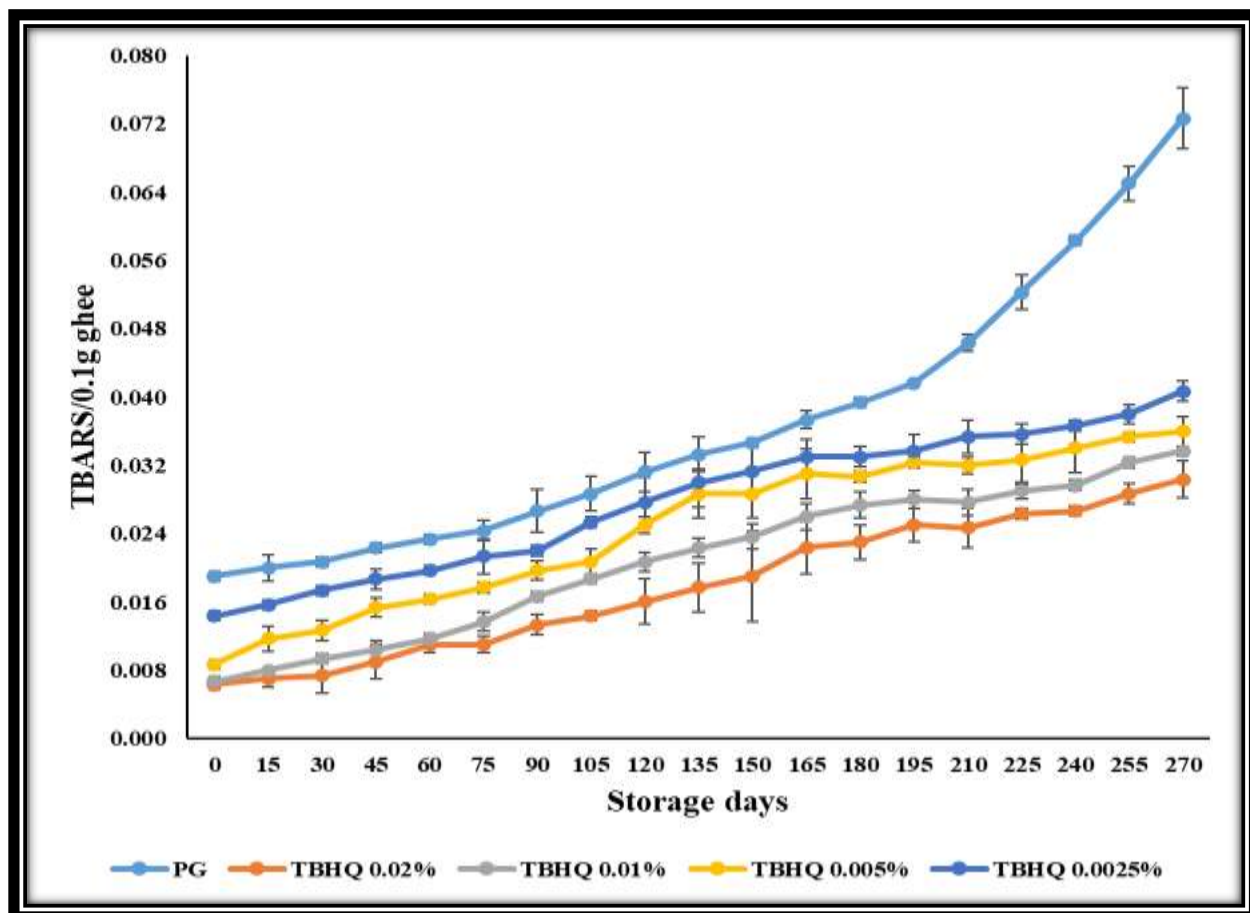


Fig 4.25 Effect of storage period on the TBA-value of cow ghee and TBHQ spiked PG samples at ambient temperature (30°C)

4.3.2.4 Effect of storage period on the TBA-value of PG and BHA spiked PG samples at ambient temperature (30°C)

These results have been depicted in Table 4.17 and Fig.4.26. It is evident from the data that PG as well as TBHQ spiked ghee samples showed an increase in the TBA value from the 0 days onwards. It was observed that in TBHQ spiked ghee samples, the TBA value significantly increased ($P < 0.05$) as the storage period progressed and increase was corresponding to the added TBHQ in the ghee. The TBA value of control PG significantly increased ($P < 0.05$) from 0.019 ± 0.001 on 0 days to 0.073 ± 0.004 after 9 months (270 days). This increase was lower than those of TBHQ spiked PG samples in the order Va, Vb, Vc and Vd (0.052 ± 0.001 , 0.056 ± 0.003 , 0.060 ± 0.002 , 0.070 ± 0.001), respectively.

The result in Table 4.25 illustrated that the treatment i.e., BHA spiked ghee samples had significant effect on the TBA value similarly, the storage days had also significant effect on TBA value. The interaction effect between treatment and storage days on the development of TBA was significant at 95% level of confidence with p -value < 0.001 . The PG sample highest increase in TBA value may be due to the more secondary oxidation products formed during storage. Similar reason drawn when they added BHA and natural antioxidant like orange peel extract in ghee Asa et al., (2015), BHA incorporated into ghee Rahila (2016).

Rahila (2016) also reported that the peroxide value of cow ghee added with BHA (synthetic antioxidant) during storage was increased for 1-210 days of storage at $37 \pm 1^\circ\text{C}$ and 1-60 days of storage at $60 \pm 1^\circ\text{C}$ from (0.00-19.69) and (0.00-23.02) mM O_2/kg of ghee, respectively.

Table 4.17 Effect of storage period on the TBA-value of PG and BHA spiked PG samples at ambient temperature (30°C)

Storage Days	TBA-value (TBARS/0.1g ghee)				
	PG Pure	Va	Vb	Vc	Vd
0	0.019±0.001 ^{aB}	0.010±0.001 ^{aA}	0.014±0.001 ^{aB}	0.015±0.001 ^{aB}	0.016±0.001 ^{aB}
15	0.020±0.002 ^{bC}	0.011±0.001 ^{aA}	0.015±0.002 ^{bB}	0.017±0.001 ^{bBC}	0.017±0.001 ^{bBC}
30	0.021±0.001 ^{cC}	0.012±0.001 ^{bA}	0.016±0.001 ^{cB}	0.018±0.001 ^{bB}	0.019±0.001 ^{cBC}
45	0.022±0.001 ^{cdC}	0.015±0.002 ^{cA}	0.018±0.002 ^{cB}	0.020±0.001 ^{cB}	0.021±0.002 ^{cdBC}
60	0.023±0.001 ^{dC}	0.016±0.001 ^{cdA}	0.020±0.002 ^{dB}	0.021±0.001 ^{cB}	0.021±0.001 ^{deB}
75	0.024±0.001 ^{dC}	0.017±0.001 ^{cdA}	0.021±0.002 ^{deB}	0.022±0.001 ^{dB}	0.023±0.001 ^{eB}
90	0.026±0.003 ^{eD}	0.018±0.000 ^{dA}	0.023±0.003 ^{efB}	0.024±0.001 ^{eBC}	0.025±0.001 ^{fC}
105	0.028±0.002 ^{fD}	0.019±0.001 ^{eA}	0.024±0.002 ^{fB}	0.027±0.001 ^{fC}	0.028±0.001 ^{gC}
120	0.031±0.002 ^{gD}	0.020±0.001 ^{eA}	0.027±0.002 ^{gB}	0.029±0.001 ^{gC}	0.030±0.002 ^{hC}
135	0.033±0.002 ^{gD}	0.022±0.001 ^{fA}	0.030±0.001 ^{hB}	0.032±0.001 ^{hBC}	0.032±0.001 ^{iCD}
150	0.035±0.001 ^{hE}	0.023±0.002 ^{fA}	0.031±0.001 ^{hiB}	0.033±0.002 ^{hC}	0.034±0.001 ^{jD}
165	0.037±0.001 ^{iD}	0.025±0.001 ^{gA}	0.033±0.001 ^{iB}	0.034±0.001 ^{iB}	0.036±0.002 ^{kC}
180	0.039±0.001 ^{jD}	0.028±0.001 ^{hA}	0.036±0.001 ^{jB}	0.037±0.001 ^{jB}	0.038±0.001 ^{lC}
195	0.042±0.002 ^{kE}	0.031±0.001 ^{iA}	0.037±0.003 ^{jB}	0.039±0.001 ^{kC}	0.040±0.001 ^{mD}
210	0.046±0.003 ^{lD}	0.032±0.002 ^{jA}	0.043±0.002 ^{kB}	0.045±0.001 ^{lB}	0.045±0.001 ^{nC}
225	0.052±0.002 ^{mE}	0.038±0.001 ^{kA}	0.046±0.003 ^{lB}	0.049±0.002 ^{mC}	0.050±0.002 ^{oD}
240	0.058±0.002 ^{nE}	0.044±0.001 ^{lA}	0.048±0.001 ^{mB}	0.053±0.001 ^{nC}	0.055±0.001 ^{pD}
255	0.065±0.003 ^{oE}	0.047±0.001 ^{mA}	0.050±0.002 ^{nB}	0.055±0.001 ^{oC}	0.063±0.001 ^{qD}
270	0.073±0.004 ^{pE}	0.052±0.001 ^{nA}	0.056±0.003 ^{oB}	0.060±0.002 ^{pc}	0.070±0.001 rD

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; Va: PG spiked with BHA @ 0.02%; Vb: PG spiked with BHA @ 0.01%; Vc: PG spiked with BHA @ 0.005%; Vd: PG spiked with BHA @ 0.0025%

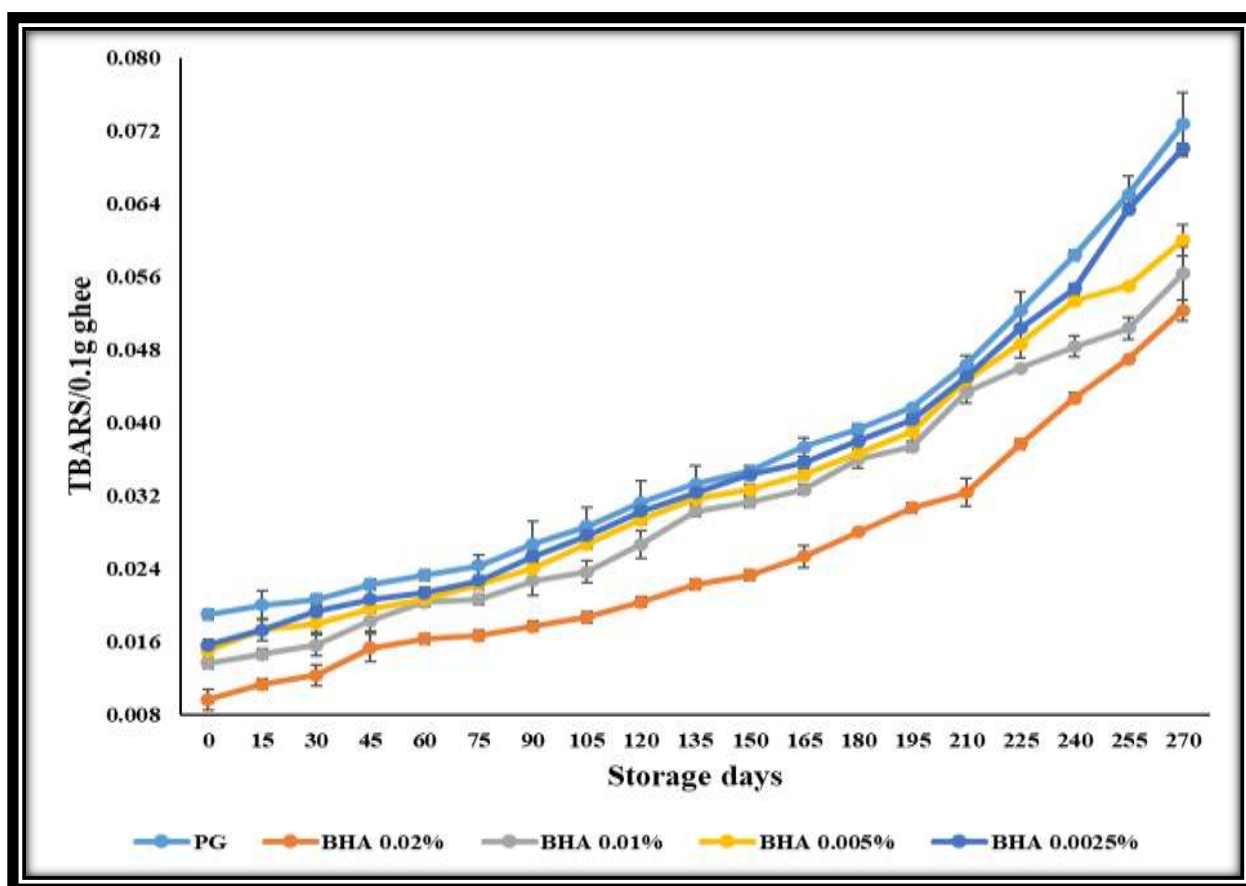


Fig 4.26 Effect of storage period on the TBA-value of cow ghee and BHA spiked PG samples at ambient temperature (30°C)

4.3.3 Evaluation of sensitivity of sensor in stored pure ghee sample and adulterated ghee samples

Antioxidants were extracted as per the standardized protocol in section 3.2.3.1 after every 15 days interval up to 9 months. The sensor was evaluated for its sensitivity using the extracted antioxidants in stored pure ghee sample and adulterated ghee samples.

4.3.3.1 Response of DPPH impregnated paper-based disc sensor in samples (PG, PO and PO spiked PG) stored at ambient temperature (30°C)

The response time of DPPH impregnated paper-based disc sensor was determined and expressed in min. It is evident from the results (Table 4.18 and Fig 4.27) that in stored PO sample the response time of DPPH impregnated paper-based disc sensor remained unaltered till 135 days of storage at ambient temperature. It is also evident from the data (Table 4.18) that palm oil had the response time (0.50 min) on 0 day of storage. It was also observed that the response time increased to 1.0 min even at the end of storage of 270 days. The response time of the sensor in control PG significantly increased ($P < 0.05$) from 250.00 ± 5.00 min on 0 day to 416.00 ± 5.29 min after 9 months (270 days), whereas, response time of PO was increased to 1.0 on 270 days of the storage. It is also evident from the results that the response time in all the samples spiked with PO upto 2.5% was lower than the response time of the sensor in PG. On the contrary, in case of ghee samples spiked with PO @1% the response time of the sensor was 246.67 min on 270th day of storage, which was very close to the response time of the sensor observed in PG on 0 day. These findings led to the conclusion that on account of storage there may be the degradation of the added antioxidant/ utilization of the added antioxidant to counter the oxidation process. This exhaustion of the antioxidant may be responsible for the increase in the response time of the sensor in stored ghee samples as well as stored PO spiked ghee samples.

The statistical analysis of the data (Table 4.22) indicated that the treatment i.e., palm oil spiked ghee samples had significant effect on the response time of DPPH impregnated paper-based disc sensor. Similarly, storage days also showed a significant effect on the response time of DPPH impregnated paper-based disc sensor. The interaction effect between treatment and storage days on the response time was affected significantly at 95% level of confidence with p -value < 0.001 Table 4.22. The response time of DPPH impregnated paper-based disc sensor was significantly high in control PG than PO spiked PG samples and PO.

Table 4.18 Response time of DPPH impregnated paper-based disc sensor in stored PG, PO and PO spiked PG samples at ambient temperature (30°C)

Storage Days	Response time (min)						
	PG	PO	Si	Sii	Siii	Siv	Sv
0	250.00±5.00 ^{aD}	0.50 ^{aA}	3.00±0.50 ^{aA}	5.00±0.50 ^{aA}	8.67±0.58 ^{aA}	20.33±0.58 ^{aB}	143.33±5.55 ^{aC}
15	250.00±5.00 ^{aE}	0.50 ^{aA}	3.00±0.50 ^{aAB}	5.00±0.50 ^{aAB}	9.33±0.58 ^{aB}	20.67±0.58 ^{aC}	153.33±2.89 ^{abD}
30	265.00±5.00 ^{bF}	0.50 ^{aA}	3.00±0.50 ^{abAB}	5.67±0.58 ^{abB}	10.33±0.58 ^{bC}	29.67±0.58 ^{bD}	158.33±2.89 ^{abE}
45	276.67±5.77 ^{cF}	0.50 ^{aA}	3.00±0.50 ^{abcAB}	5.33±0.58 ^{abcB}	10.67±0.58 ^{bC}	34.00±2.00 ^{cd}	166.67±2.89 ^{bce}
60	285.00±5.00 ^{cdF}	0.50 ^{aA}	3.33±0.58 ^{abcdAB}	5.67±0.58 ^{abcB}	11.67±0.58 ^{cdC}	41.00±1.73 ^{deD}	170.00±5.00 ^{bcdE}
75	288.33±2.89 ^{dF}	0.50 ^{aA}	3.67±0.58 ^{abcdB}	5.67±0.58 ^{abcB}	11.67±0.58 ^{cdC}	42.00±1.73 ^{deD}	178.33±2.89 ^{cdE}
90	293.33±5.77 ^{deF}	0.50 ^{aA}	4.00±0.50 ^{abcdAB}	6.00±0.50 ^{bcdB}	11.67±0.58 ^{cdC}	44.33±2.52 ^{deD}	188.33±2.89 ^{deE}
105	300.00±5.00 ^{eF}	0.50 ^{aA}	4.00±0.50 ^{abcdAB}	6.33±0.58 ^{cdB}	12.33±0.58 ^{cdC}	48.00±1.00 ^{deD}	198.33±2.89 ^{efE}
120	300.00±5.00 ^{fE}	0.50 ^{aA}	4.00±0.50 ^{abcdA}	6.33±0.58 ^{cdAB}	12.33±0.58 ^{cdB}	49.67±1.58 ^{deC}	205.00±5.00 ^{efD}
135	325.00±5.00 ^{gF}	0.50 ^{aA}	4.00±0.50 ^{abcdAB}	6.67±0.58 ^{dB}	12.33±0.58 ^{cdC}	52.00±1.00 ^{deD}	211.67±2.89 ^{fgE}
150	337.67±2.52 ^{hF}	1.00 ^{aA}	4.00±0.50 ^{abcdB}	6.67±0.58 ^{dB}	12.33±0.58 ^{cdC}	54.00±1.00 ^{ghD}	216.33±4.04 ^{ghE}
165	353.33±5.77 ^{iD}	1.00 ^{aA}	4.67±0.58 ^{bcdA}	7.67±0.58 ^{eA}	12.67±0.58 ^{cdA}	56.00±1.00 ^{hiB}	220.33±4.81 ^{ghiC}
180	360.00±5.00 ^{iF}	1.00 ^{aA}	4.67±0.58 ^{cdAB}	8.00±0.50 ^{eB}	13.00±1.00 ^{deC}	57.67±1.58 ^{ijD}	225.00±5.00 ^{hijE}
195	373.33±2.89 ^{jG}	1.00 ^{aA}	4.67±0.58 ^{cdB}	8.00±0.50 ^{ec}	13.33±1.58 ^{defD}	59.00±1.00 ^{jke}	228.67±5.89 ^{hijF}
210	383.33±2.89 ^{kG}	1.00 ^{aA}	5.00±0.50 ^{dB}	8.00±0.50 ^{ec}	13.67±1.58 ^{efgD}	60.33±1.58 ^{kIE}	230.33±5.15 ^{ijkF}
225	391.67±2.89 ^{klG}	1.00 ^{aA}	5.00±0.50 ^{dB}	8.00±0.50 ^{ec}	14.00±1.00 ^{ghiD}	60.33±1.15 ^{kIE}	233.00±5.73 ^{jkF}
240	395.00±5.00 ^{lmF}	1.00 ^{aA}	5.00±0.50 ^{dB}	8.00±0.50 ^{eb}	14.33±1.58 ^{hiC}	61.33±1.15 ^{kID}	236.33±5.15 ^{jke}
255	404.00±5.29 ^{mF}	1.00 ^{aA}	5.00±0.50 ^{dAB}	8.00±1.00 ^{eb}	14.67±1.58 ^{ijC}	62.00±1.73 ^{lD}	241.67±5.89 ^{jke}
270	416.00±5.29 ^{nF}	1.00 ^{aA}	5.00±0.50 ^{dAB}	8.00±1.00 ^{eb}	15.33±1.58 ^{jc}	65.00±2.00 ^{mD}	246.67±5.89 ^{ke}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; PO: Palm Oil; Si: PG spiked with PO @ 10%; Sii: PG spiked with PO @ 7.5%; Siii: PG spiked with PO @ 5%; Siv: PG spiked with PO @ 2.5%; Sv: PG spiked with PO @ 1%;

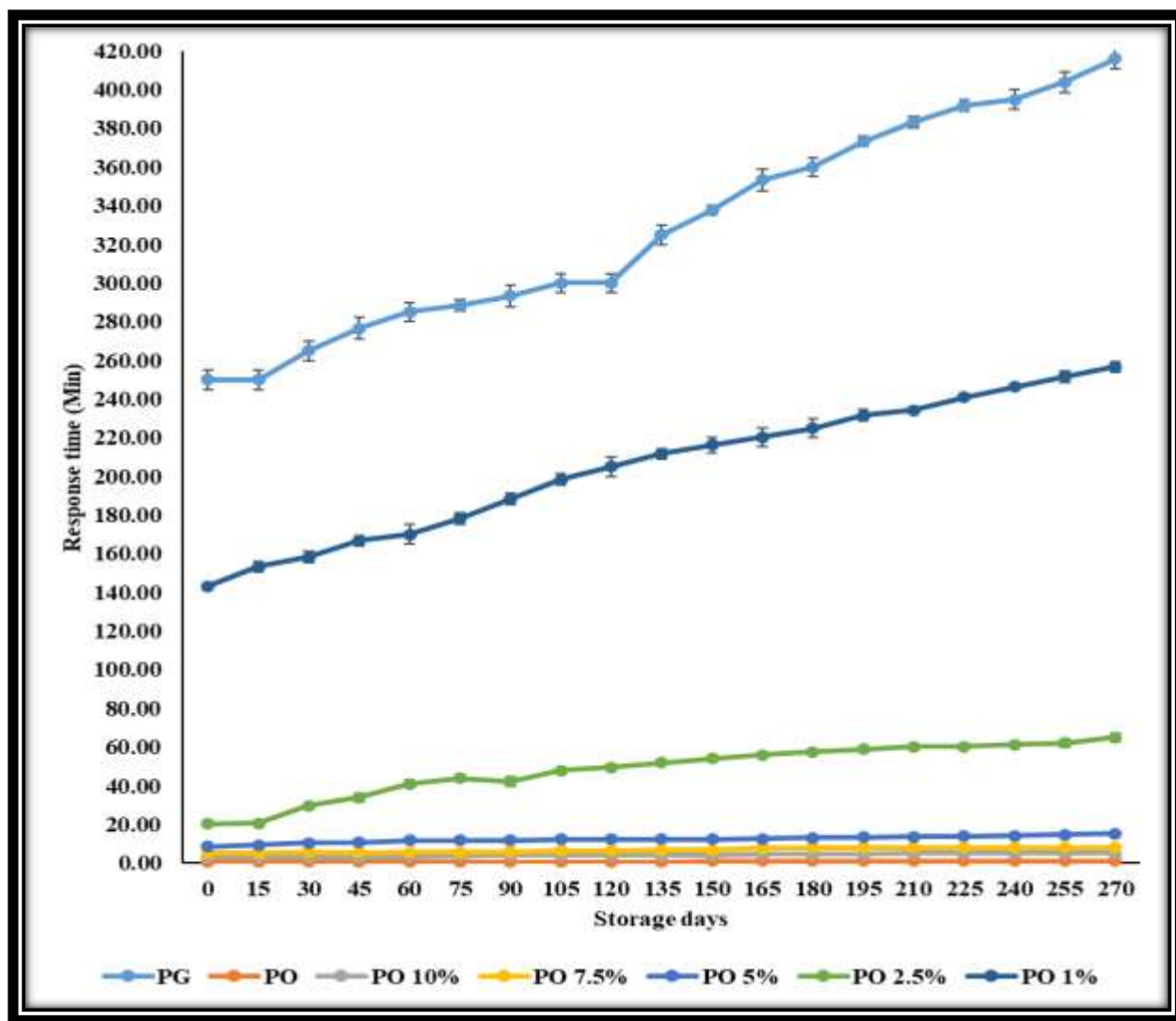


Fig.4.27 Response of DPPH impregnated paper-based disc sensor in stored PG, PO and PO spiked PG at ambient temperature (30°C)

4.3.3.2 Response of DPPH impregnated paper-based disc sensor in stored PG, SFO and SFO spiked PG at ambient temperature (30°C)

The response time of DPPH impregnated paper-based disc sensor was determined and expressed in min. These results have been depicted in Table 4.19 and Fig 4.28. It is evident from the results that in stored SFO sample, the response time of DPPH impregnated paper-based disc sensor was remained unaltered till 120 days of storage at ambient temperature. It is also evident from the data (Table 4.19) that in case of SFO the response time noted was 0.50 min on zero day of storage. It was also observed that in spiked ghee samples, the response time significantly increased ($P<0.05$) and was found to be more than the pure SFO. This could be attributed to the dilution of the antioxidant present in SFO, when it was spiked in pure ghee samples. As the storage period progressed an increase in the response time was observed in all the samples including pure ghee as well as SFO. The response time of control PG significantly increased ($P<0.05$) from 250.00 ± 5.00 on 0 days to 416.00 ± 5.29 after 9 months (270 days), whereas, response time of SFO was increased to 1.33 on 270 days of the storage. In the ghee samples spiked with SFO, it was observed that there was an increase in the response time on account of spiking rate up to 2.5% as well as storage period. However, the response time recorded in these spiked and stored samples was less than 30 min. It was also observed that in case of samples spiked with SFO @ 1% the response time increased to 156 min at the 270th day of storage. From the results it can be concluded that the comfortable level which could be detected even in stored ghee samples was 2.5%.

The result in Table 4.23 indicated that the treatment i.e., sunflower oil spiked ghee samples had significant effect on the peroxide value, similarly storage days had also significant effect on the peroxide value. The interaction effect between treatment and storage days on the response time was affected significantly at 95% level of confidence with p - value <0.001 Table 4.23. The response time of DPPH impregnated paper-based disc sensor was significantly developed in PG than SFO spiked ghee samples and SFO. It was due to the fact that the max permitted antioxidant 0.02% as per FSSAI available in SFO and for control PG not permitted any kind of antioxidants. The antioxidants were donating the hydrogen atom or DPPH accept the electron and reduce its color from violet to yellow color (Liu *et al.*, 2014).

Table 4.19 Response time of DPPH impregnated paper-based disc sensor in stored PG, SFO and SFO spiked PG samples at ambient temperature (30°C)

Storage Days	Response time (min)						
	PG	SFO	Ti	Tii	Tiii	Tiv	Tv
0	250.00±5.00 ^{aE}	0.50 ^{aA}	3.00±0.25 ^{aAB}	5.00±0.50 ^{aAB}	9.33±0.58 ^{aB}	19.00±1.0 ^{aC}	60.67±1.15 ^{aD}
15	250.00±5.00 ^{aE}	0.50 ^{aA}	3.00±0.25 ^{aAB}	5.00±0.50 ^{aAB}	9.67±0.58 ^{aB}	19.00±1.0 ^{aC}	63.00±1.73 ^{aD}
30	265.00±5.00 ^{bF}	0.50 ^{aA}	3.00±0.25 ^{aAB}	6.33±0.58 ^{bBC}	9.67±0.58 ^{abC}	19.67±0.58 ^{aD}	69.00±1.73 ^{bE}
45	276.67±5.77 ^{cF}	0.50 ^{aA}	3.67±0.58 ^{abAB}	6.33±0.58 ^{bBC}	9.67±0.58 ^{abC}	19.00±1.00 ^{aD}	74.33±1.15 ^{cE}
60	285.00±5.00 ^{cdF}	0.50 ^{aA}	3.67±0.58 ^{abAB}	6.33±0.58 ^{bBC}	9.67±0.58 ^{abC}	22.33±1.53 ^{aD}	78.00±1.73 ^{dE}
75	288.33±2.89 ^{dG}	0.50 ^{aA}	3.67±0.58 ^{abB}	6.67±0.58 ^{bC}	9.67±0.58 ^{abD}	20.00±1.0 ^{bE}	83.00±1.73 ^{eF}
90	293.33±5.77 ^{de}	0.50 ^{aA}	4.00±0.50 ^{bcAB}	6.67±0.58 ^{bcBC}	10.00±0.50 ^{abC}	22.33±0.58 ^{bD}	88.67±1.15 ^{fE}
105	300.00±5.00 ^{eF}	0.50 ^{aA}	4.00±0.50 ^{bcAB}	6.67±0.58 ^{bcB}	10.67±0.58 ^{abcC}	23.33±0.58 ^{bcD}	93.33±2.89 ^{gE}
120	300.00±5.00 ^{fF}	0.50 ^{aA}	4.00±0.50 ^{bcAB}	6.67±0.58 ^{bcAB}	10.67±0.58 ^{abcB}	23.33±0.58 ^{bcC}	95.67±2.15 ^{gD}
135	325.00±5.00 ^{gE}	0.67±0.29 ^{aA}	4.33±0.58 ^{bcdAB}	7.33±0.58 ^{bcdBC}	11.00±1.00 ^{bcC}	23.33±1.53 ^{bcdD}	99.00±2.73 ^{hE}
150	337.67±2.52 ^{hFG}	0.67±0.29 ^{aA}	4.33±0.58 ^{bcdB}	7.33±0.58 ^{bcdC}	11.00±1.00 ^{bcdD}	24.33±1.58 ^{cdE}	103.67±2.15 ^{iF}
165	353.33±5.77 ^{iF}	1.00±0.25 ^{bA}	4.67±0.58 ^{cdeAB}	7.67±0.58 ^{cdeBC}	11.67±1.53 ^{cdC}	24.67±1.58 ^{cdD}	108.00±2.73 ^{jE}
180	360.00±5.00 ^{iF}	1.00±0.25 ^{bA}	4.67±0.58 ^{cdeB}	7.67±0.58 ^{cdeB}	12.33±1.15 ^{deC}	25.00±1.50 ^{deD}	113.00±2.73 ^{kE}
195	373.33±2.89 ^{jG}	1.00±0.25 ^{bA}	4.67±0.58 ^{cdeB}	7.67±0.58 ^{cdeC}	12.67±0.58 ^{deD}	25.33±1.58 ^{defE}	118.67±2.15 ^{iF}
210	383.33±2.89 ^{kG}	1.17±0.29 ^{bA}	5.00±0.50 ^{deB}	7.67±0.58 ^{cdeC}	13.00±0.50 ^{edD}	25.67±1.58 ^{defE}	124.33±2.58 ^{mF}
225	391.67±2.89 ^{klG}	1.17±0.29 ^{bA}	5.00±0.50 ^{deB}	8.00±1.00 ^{deC}	13.33±0.58 ^{edD}	26.33±1.58 ^{efgE}	131.33±2.15 ^{nF}
240	395.00±5.00 ^{lmF}	1.33±0.29 ^{bA}	5.33±0.58 ^{EB}	8.33±0.58 ^{deB}	13.67±0.58 ^{EC}	26.67±1.58 ^{fgD}	140.67±2.15 ^{oE}
255	404.00±5.29 ^{mF}	1.33±0.29 ^{bA}	5.33±0.58 ^{EB}	8.67±0.58 ^{EB}	13.67±0.58 ^{EC}	27.33±1.58 ^{hD}	148.33±2.15 ^{pE}
270	416.00±5.29 ^{nF}	1.33±0.29 ^{bA}	5.33±0.58 ^{EB}	8.67±0.58 ^{EB}	13.67±0.58 ^{EC}	27.67±1.58 ^{hD}	156.33±2.15 ^{qE}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; SFO: Sunflower Oil; Ti: PG spiked with SFO @ 10%; Tii: PG spiked with SFO @ 7.5%; Tiii: PG spiked with SFO @ 5%; Tiv: PG spiked with SFO @ 2.5%; Tv: PG spiked with SFO @ 1%;

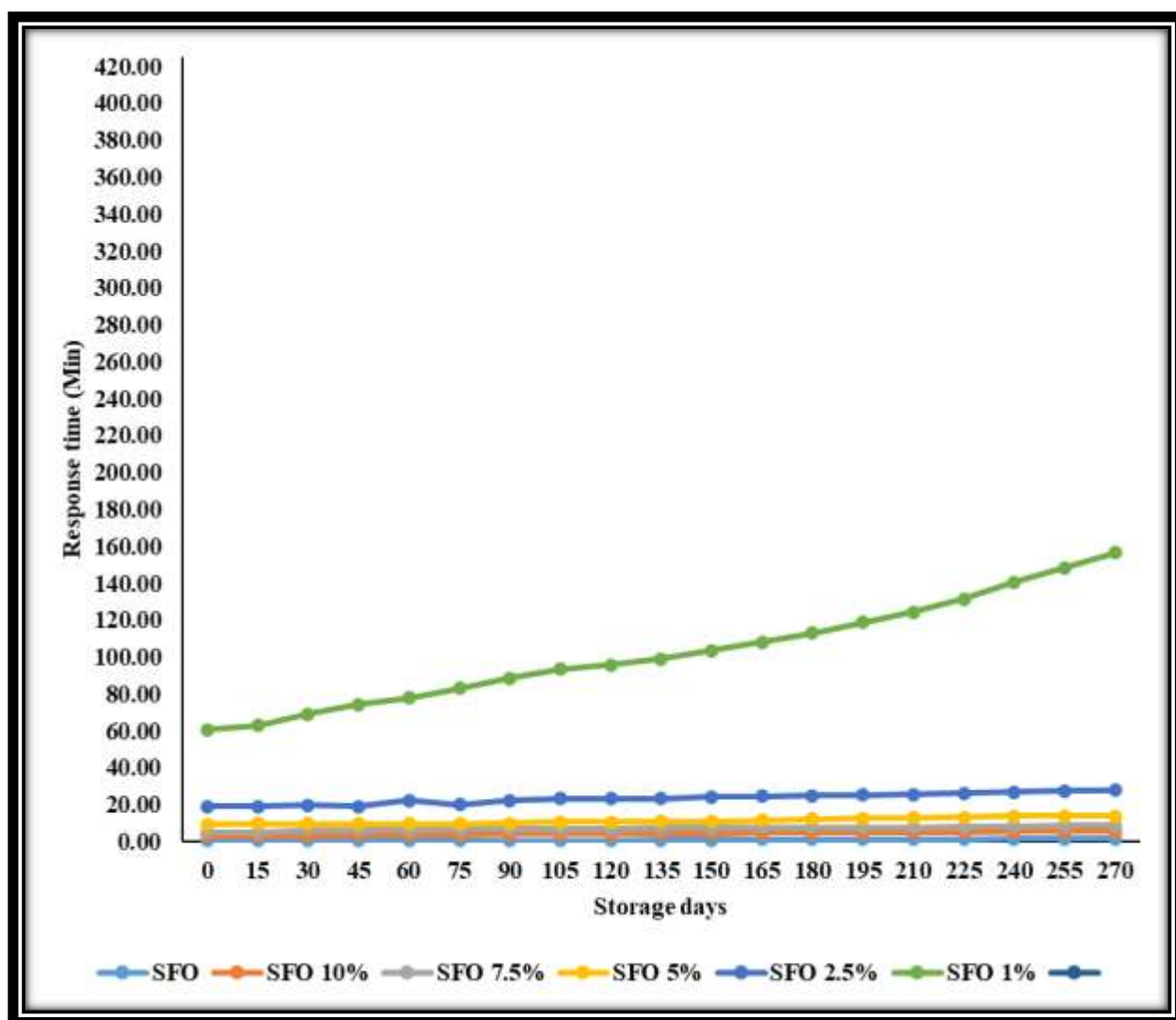


Fig 4.28 Response of DPPH impregnated paper-based disc sensor in stored PG, SFO and SFO spiked PG at ambient temperature (30°C)

palm oil and cotton seed oil adulterated ghee sample also changed DPPH violet to yellow color within 30 seconds and 10 minutes, respectively because of presence of antioxidants in the respective oils (Joshi, 2015; Ramani, 2018).

4.3.3.3 Response of DPPH impregnated paper-based disc sensor in stored PG and TBHQ spiked PG at ambient temperature (30°C)

The response time of DPPH impregnated paper-based disc sensor was determined and expressed in min. These results have been depicted in Table 4.20 and Fig 4.29. It is evident from the results that in stored TBHQ spiked ghee samples @ 0.05, 0.01 and 0.02% the response time of DPPH impregnated paper-based disc sensor was remained same till 270 days of storage at ambient temperature. On the contrary, in case of ghee samples spiked with TBHQ @ 0.0025% an increase in response time of DDH impregnated paper-based disc sensor was observed after 195 days. The response time of control PG significantly increased ($P<0.05$) from 250.00 ± 5.00 on 0 days to 416.00 ± 5.29 after 9 months (270 days).

The result in Table 4.24 illustrated that the treatment i.e., TBHQ spiked ghee samples had significant effect on the response time of DPPH impregnated paper-based disc sensor similarly the storage days had also significant effect on the response time of DPPH impregnated paper-based disc sensor. The interaction effect between treatment and storage days on the response time was affected significantly at 95% level of confidence with p -value <0.001 Table 4.24. The response time of DPPH impregnated paper-based disc sensor was significantly developed in decreasing order PG than TBHQ spiked ghee samples. The reason behind this is that the max permitted level of antioxidant TBHQ (0.02-0.0025) % in vegetable oils were added in PG except control PG. The antioxidants were donating the hydrogen atom or DPPH accept the electron and reduce its color from violet to yellow color (Liu *et al.*, 2014).

Table 4.20 Response time of DPPH impregnated paper-based disc sensor in stored PG and TBHQ spiked PG at ambient temperature (30°C)

Storage Days	Response time (min)				
	PG	Ui	Uii	Uiii	Uiv
0	250.00±5.00 ^{aB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
15	250.00±5.00 ^{aB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
30	265.00±5.00 ^{bC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aAB}	3.00±0.50 ^{aAB}	5.67±0.58 ^{aB}
45	276.67±5.77 ^{cB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
60	285.00±5.00 ^{cdC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aAB}	3.00±0.50 ^{aAB}	5.67±0.58 ^{aB}
75	288.33±2.89 ^{dC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aB}
90	293.33±5.77 ^{deB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
105	300.00±5.00 ^{eC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aAB}	3.00±0.50 ^{aAB}	5.67±0.58 ^{aB}
120	300.00±5.00 ^{tB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
135	325.00±5.00 ^{gC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aAB}	3.00±0.50 ^{aAB}	5.67±0.58 ^{aB}
150	337.67±2.52 ^{hC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aB}
165	353.33±5.77 ^{iB}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	5.67±0.58 ^{aA}
180	360.00±5.00 ^{iC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aAB}	3.00±0.50 ^{aAB}	5.67±0.58 ^{aB}
195	373.33±2.89 ^{jC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	6.33±0.58 ^{aB}
210	383.33±2.89 ^{kC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	6.33±0.58 ^{aB}
225	391.67±2.89 ^{klC}	1.00±0.25 ^{aA}	2.00±0.25 ^{aA}	3.00±0.50 ^{aA}	6.33±0.58 ^{aB}
240	395.00±5.00 ^{lmC}	1.00±0.25 ^{aA}	2.00±0.50 ^{aA}	3.00±0.50 ^{aAB}	6.33±0.58 ^{aB}
255	404.00±5.29 ^{mC}	1.00±0.25 ^{aA}	2.00±0.50 ^{aAB}	3.00±0.50 ^{aAB}	6.33±0.58 ^{aB}
270	416.00±5.29 ^{nC}	1.00±0.25 ^{aA}	2.00±0.50 ^{aAB}	3.00±0.50 ^{aAB}	6.33±0.58 ^{aB}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; Ui: PG spiked with TBHQ @ 0.02%; Uii: PG spiked with TBHQ @ 0.01%; Uiii: PG spiked with TBHQ @ 0.005%; Uiv: PG spiked with TBHQ @ 0.0025%

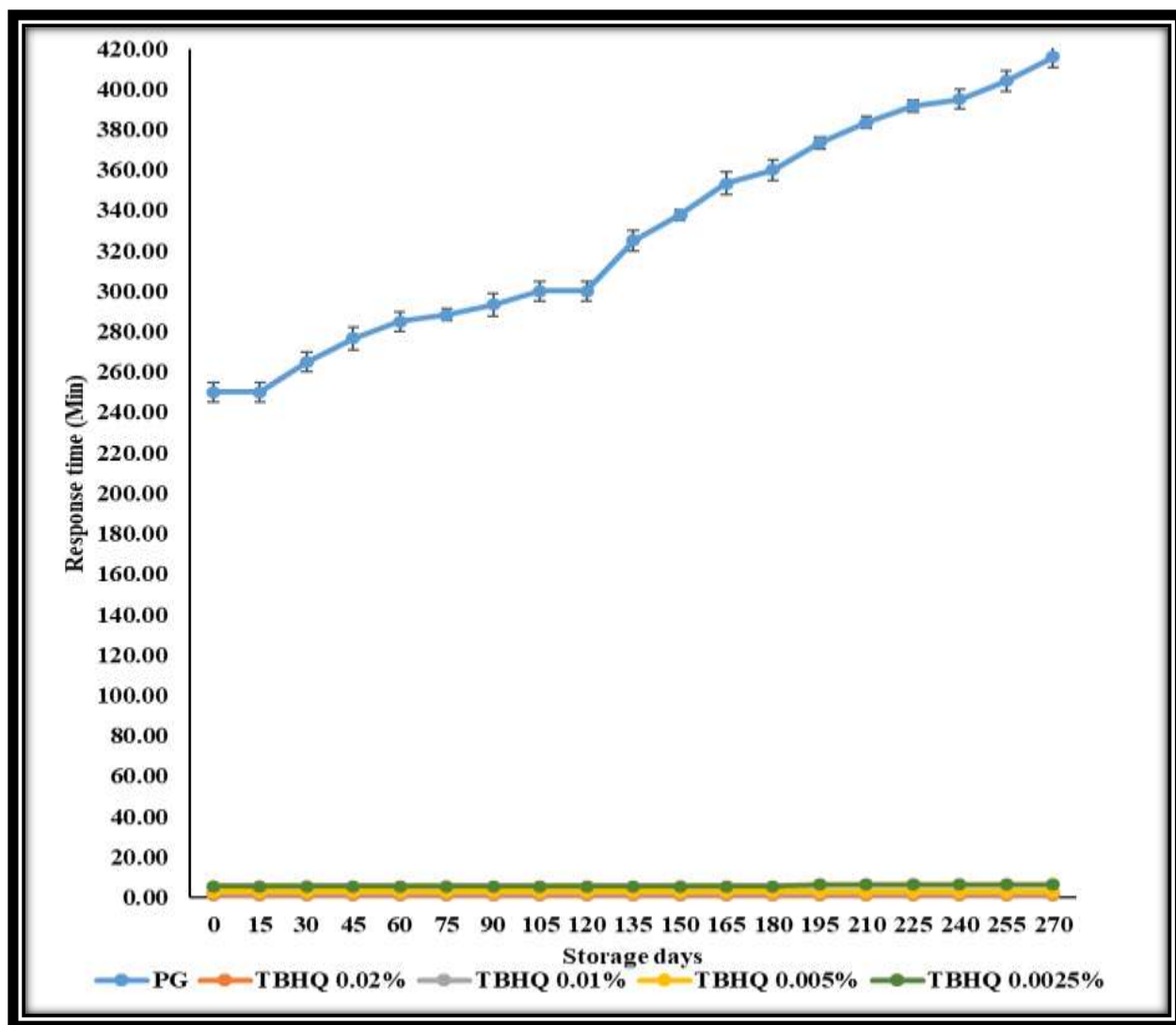


Fig 4.29 Response of DPPH impregnated paper-based disc sensor in stored PG and TBHQ spiked PG at ambient temperature (30°C)

4.3.3.4 Response of DPPH impregnated paper-based disc sensor in stored PG and BHA spiked PG samples at ambient temperature (30°C)

The response time of DPPH impregnated paper-based disc sensor was determined and expressed in min. These results have been depicted in Table 4.21 and Fig 4.30. It is evident from the results that in stored BHA spiked ghee samples @ 0.01 and 0.02% the response time of DPPH impregnated paper-based disc sensor was almost same till 45 days of storage at ambient temperature. On the contrary, in case of ghee samples spiked with BHA @ 0.0025 and 0.005% an increase in response time of DPPH impregnated paper-based disc sensor was recorded. It was also recorded that the increase in response time was more as a result of storage of such samples. This could be attributed to the lower level of the antioxidant added in such samples which might have exhausted at a faster rate during storage. The response time of control PG significantly increased ($P < 0.05$) from 250.00 ± 5.00 on 0 days to 416.00 ± 5.29 after 9 months (270 days). This increase was higher than those of BHA spiked PG samples in the order Vi, Vii, Viii and Viv (5.00 ± 1.00 , 8.67 ± 0.58 , 22.67 ± 1.58 , 47.33 ± 1.58), respectively.

The result in Table 4.25 illustrated that the treatment i.e., BHA spiked ghee samples had significant effect on the response time of DPPH impregnated paper-based disc sensor similarly the storage days had also significant effect on the response time of DPPH impregnated paper-based disc sensor. The interaction effect between treatment and storage days on the response time was affected significantly at 95% level of confidence with p -value < 0.001 Table 4.25. The response time of DPPH impregnated paper-based disc sensor was significantly developed in decreasing order PG than BHA spiked ghee samples. The reason behind this is that the max permitted level of antioxidant BHA (0.02-0.0025) % in vegetable oils were added in PG except control PG. The antioxidants were donating the hydrogen atom to DPPH or DPPH accept the electron and reduce its color from violet to yellow color (Liu *et al.*, 2014).

Table 4.21 Response time of DPPH impregnated paper-based disc sensor in stored PG and BHA spiked PG at ambient temperature (30°C)

Storage Days	Response time (min)				
	PG	Vi	Vii	Viii	Viv
0	250.00±5.00 ^{aD}	3.00±0.50 ^{aA}	6.33±0.58 ^{aAB}	12.33±0.58 ^{aB}	26.67±1.53 ^{aC}
15	250.00±5.00 ^{aD}	3.00±0.58 ^{aA}	6.33±0.58 ^{aAB}	13.00±0.50 ^{aB}	26.67±1.15 ^{aC}
30	265.00±5.00 ^{bD}	3.00±0.50 ^{aA}	6.33±0.58 ^{aA}	13.00±0.50 ^{abB}	27.00±1.00 ^{aC}
45	276.67±5.77 ^{cD}	3.00±0.50 ^{aA}	6.33±0.58 ^{aA}	13.67±0.58 ^{abB}	27.00±1.00 ^{aC}
60	285.00±5.00 ^{cdD}	3.67±0.58 ^{abA}	6.67±0.58 ^{abA}	13.67±0.58 ^{abB}	27.00±1.00 ^{aC}
75	288.33±2.89 ^{dE}	3.67±0.58 ^{abA}	6.67±0.58 ^{abcB}	13.67±0.58 ^{abcC}	27.00±1.00 ^{aD}
90	293.33±5.77 ^{deD}	3.67±0.58 ^{abA}	7.00±0.58 ^{abcA}	13.33±0.58 ^{abcB}	27.00±1.00 ^{aC}
105	300.00±5.00 ^{eD}	3.67±0.58 ^{abA}	7.00±0.58 ^{abcA}	13.33±0.58 ^{abcB}	27.00±1.00 ^{aC}
120	300.00±5.00 ^{fD}	4.00±0.50 ^{abcA}	7.00±0.58 ^{abcAB}	13.33±0.58 ^{abcB}	27.00±1.00 ^{aC}
135	325.00±5.00 ^{gD}	4.00±0.50 ^{abcA}	7.00±0.58 ^{abcA}	13.33±0.58 ^{abcB}	27.00±1.00 ^{aC}
150	337.67±2.52 ^{hE}	4.00±0.50 ^{abcA}	7.00±0.58 ^{abcB}	13.33±0.58 ^{abcC}	27.00±1.00 ^{aD}
165	353.33±5.77 ^{iD}	4.33±0.58 ^{bcA}	7.33±0.58 ^{abcA}	14.33±0.58 ^{bcdB}	27.67±1.53 ^{abC}
180	360.00±5.00 ^{iD}	4.33±0.58 ^{bcA}	7.33±0.58 ^{abcA}	14.67±1.15 ^{cdB}	29.33±1.15 ^{bC}
195	373.33±2.89 ^{jE}	4.33±0.58 ^{bcA}	7.33±0.58 ^{abcB}	14.67±1.15 ^{cdC}	31.33±1.15 ^{cd}
210	383.33±2.89 ^{KE}	4.67±0.58 ^{bcA}	7.67±0.58 ^{bcdB}	15.33±1.15 ^{dC}	34.33±0.58 ^{dD}
225	391.67±2.89 ^{kIE}	4.67±0.58 ^{bcA}	8.00±1.00 ^{cdeB}	17.33±1.15 ^{eC}	37.33±1.15 ^{eD}
240	395.00±5.00 ^{lmD}	5.00±1.00 ^{cA}	8.33±0.58 ^{deA}	20.00±1.15 ^{fB}	42.00±2.00 ^{fC}
255	404.00±5.29 ^{mD}	5.00±1.00 ^{cA}	8.67±0.58 ^{eA}	21.33±1.15 ^{gB}	45.00±1.00 ^{gC}
270	416.00±5.29 nD	5.00±1.00 ^{cA}	8.67±0.58 ^{eA}	22.67±1.58 ^{hB}	47.33±1.58 ^{hC}

Values are represented as Mean ± SD, n=3; ^{a, b & A, B} – different superscript row-wise and column-wise, respectively differ significantly (p<0.05)

PG: Pure Ghee; Vi: PG spiked with BHA @ 0.02%; Vii: PG spiked with BHA @ 0.01%; Viii: PG spiked with BHA @ 0.005%; Viv: PG spiked with BHA @ 0.0025%

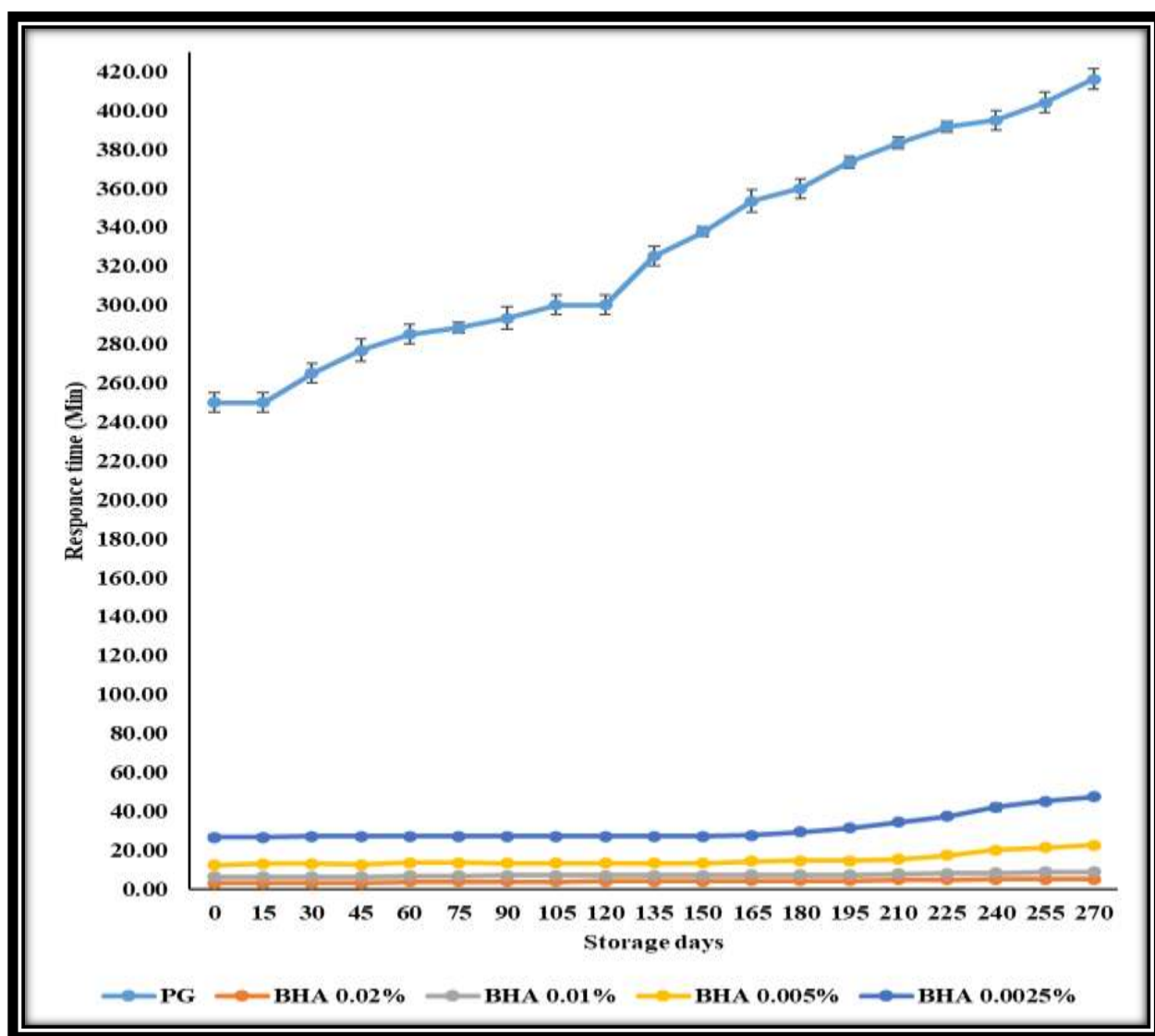


Fig 4.30 Response of DPPH impregnated paper-based disc sensor in stored PG and BHA spiked PG at ambient temperature (30°C)

Table 4.22 Two-way ANNOVA table of pure ghee, Palm oil and ghee spiked with PO different levels

Source	Peroxide Value				TBA Value				Disc color change time			
	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.
Treatment(T)	6	3384.881	6299.289	.000	.011	6	.002	.000	6	958195.042	42324.789	.000
Days(D)	18	1295.597	2411.115	.000	.077	18	.004	.000	18	4957.880	218.996	.000
TxD	108	3.739	6.959	.000	108	2.535E-005	21.075	.000	108	1427.974	63.076	.000
Error	266	.537			266	1.203E-006			266	22.639		
Total	399				399				399			

Table 4.23 Two-way ANNOVA table of pure ghee, Sunflower oil and ghee spiked with SFO different levels

Source	Peroxide Value				TBA Value				Disc color change time			
	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.
Treatment(T)	6	2360.027	5523.837	.000	6	.151	63926.438	.000	6	826476.854	152474.518	.000
Days(D)	18	1163.189	2722.539	.000	18	.014	5747.619	.000	18	3420.606	631.059	.000
TxD	108	2.294	5.370	.000	108	.001	253.743	.000	108	1328.753	245.138	.000
Error	266	.427			266	2.361E-006			266	5.420		
Total	399				399				399			

Table 4.24 Two-way ANNOVA table of pure ghee and ghee spiked with TBHQ at different levels

Source	Peroxide Value				TBA Value				Disc color change time			
	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.
Treatment(T)	4	431.139	2232.459	.000	4	.004	2507.606	.000	4	1214573.421	176970.054	.000
Days(D)	18	295.079	1527.934	.000	18	.002	1012.735	.000	18	1797.845	261.956	.000
TxD	72	10.380	53.750	.000	72	8.931E-005	58.115	.000	72	1776.332	258.821	.000
Error	190	.193			190	1.537E-006			190	6.863		
Total	285				285				285			

Table 4.25 Two-way ANNOVA table of pure ghee and ghee spiked with BHA at different levels

Source	Peroxide Value				TBA Value				Disc color change time			
	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.	Df	Mean Square	F	Sig.
Treatment(T)	4	173.994	679.662	.000	4	.002	1555.047	.000	4	1137573.833	155719.761	.000
Days(D)	18	452.487	1767.529	.000	18	.003	3290.711	.000	18	1797.845	261.956	.000
TxD	72	4.392	17.158	.000	72	2.826E-005	27.772	.000	72	1649.746	225.830	.000
Error	190	.256			190	1.018E-006			190	7.305		
Total	285				285				285			

4.3.4 Effect of storage on the status of antioxidants in ghee samples spiked with antioxidants:

The result depicted in standardized method of GLC analysis in Fig.4.31 indicated that the PG spiked with TBHQ 0.02, 0.01, 0.005 and 0.0025% the observed concentration (green, blue, brown and purple) on 0 days of storage at ambient temperature was 0.0212 ± 0.00002 , 0.0112 ± 0.00001 , 0.0053 ± 0.00002 and $0.0026 \pm 0.00003\%$, respectively. After 270 days of storage the concentration in the same samples was reduced to 0.0207 ± 0.00001 , 0.0093 ± 0.00002 , 0.0047 ± 0.00002 and $0.0023 \pm 0.00002\%$, respectively (brown, pink, blue and brick-red).

The result depicted in standardized method of GLC analysis in Fig.4.32 indicated that the PG spiked with BHA concentration from 0.02, 0.01, 0.005 and 0.0025% the observed concentration and color (green, blue, brown and purple) on 0 days of storage at ambient temperature was 0.019 ± 0.00001 , 0.0097 ± 0.00002 , 0.0048 ± 0.00002 -and $0.0024 \pm 0.00001\%$, respectively. After 270 days of storage the same samples antioxidant concentration was reduced with color (brown, pink, blue and brick-red) to 0.017 ± 0.00001 , 0.0093 ± 0.00001 , 0.0043 ± 0.00002 and $0.0022 \pm 0.00003\%$, respectively. Which was clearly indicated that during storage of ghee samples spiked with synthetic antioxidants such as TBHQ and BHA reduced gradually at ambient temperature(30°C).

4.3.5 Effect of storage on the status of antioxidants in SFO and ghee samples spiked with vegetable oils as determined using standardized GLC conditions:

It is evident from the findings (Table 4.26 and Fig. 4.33) that SFO had the antioxidant concentration (0.013 ± 0.00002) on zero day of storage. The synthetic antioxidant level of SFO was gradually reduced or degraded to 0.010 ± 0.00008 after 210 days of storage at ambient temperature. In case of SFO spiked samples the concentration of the antioxidant was too low because of the dilution effect, when SFO was spiked in pure ghee. The level of antioxidant in the spiked ghee samples showed almost no change during storage, which may be attributed to the fact that the level was seems to be too low to study the reduction in the level on account of storage. It was observed that the GLC conditions used were capable to detect the reduction in antioxidants level in ghee, when pure / standard antioxidants were used in the study. However, in case of ghee samples spiked with vegetable oils, the residual antioxidants were too low to study using GLC.

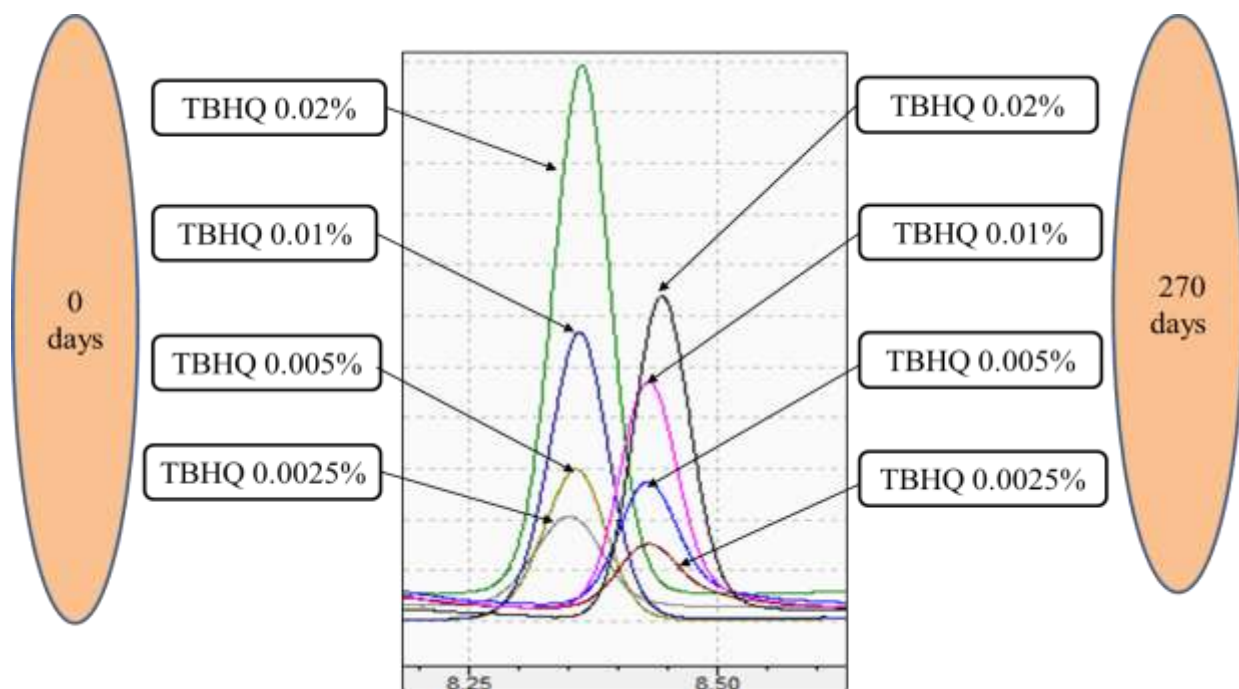


Fig. 4.32 Reduction of antioxidants in stored TBHQ spiked cow ghee samples from 0 to 270 days at ambient temperature (30°C)

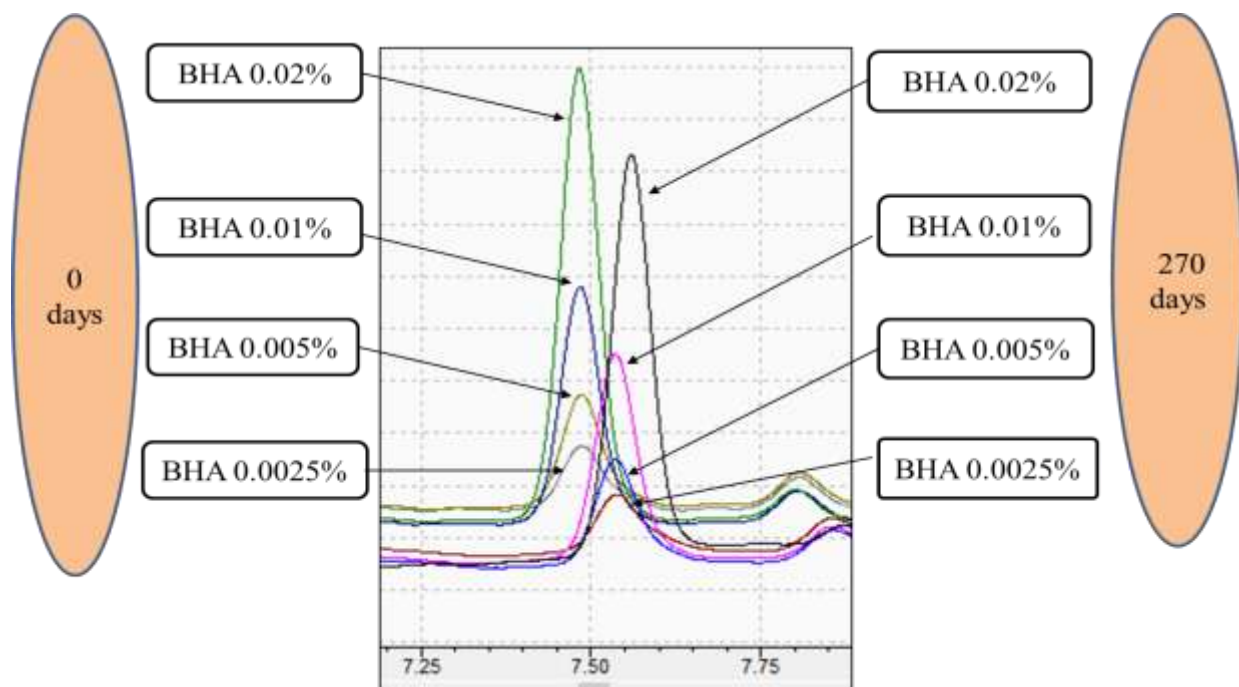


Fig. 4.33 Reduction of antioxidants in stored BHA spiked cow ghee samples from 0 to 270 days at ambient temperature (30°C)

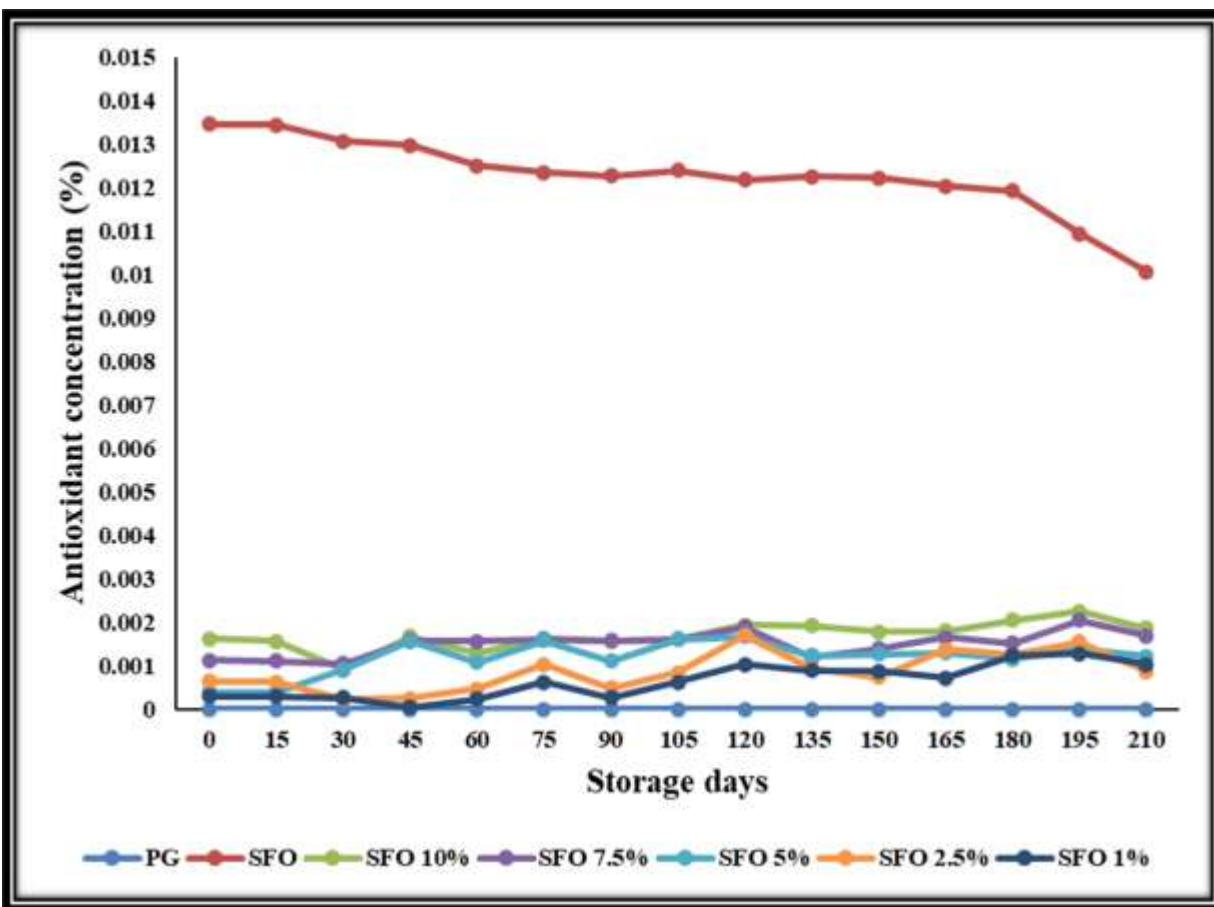


Fig. 4.33 Reduction of antioxidants in stored PG, SFO and SFO spiked ghee samples at ambient temperature (30°C)

Table 4.26 Reduction of antioxidants in stored PG, SFO and SFO spiked PG samples at ambient temperature (30°C)

Storage Days	Reduction of Antioxidant concentration (%)						
	PG	SFO	Wa	Wb	Wc	Wd	We
0	0	0.013±0.00002 ^A	0.0016±0.00001 ^C	0.0012±0.00002 ^B	0.0004±0.00002 ^A	0.0006±0.00003 ^C	0.0002±0.00001 ^A
15	0	0.013±0.00003 ^A	0.0016±0.00004 ^C	0.0012±0.00003 ^B	0.0004±0.00002 ^A	0.0006±0.00003 ^C	0.0002±0.00001 ^A
30	0	0.013±0.00002 ^A	0.0009±0.00006 ^A	0.0010±0.00002 ^A	0.0010±0.00002 ^B	0.0002±0.00001 ^A	0.0002±0.00002 ^A
45	0	0.013±0.00004 ^B	0.0017±0.00002 ^C	0.0016±0.00003 ^D	0.0016±0.00002 ^A	0.0002±0.00002 ^A	0.0002±0.00002 ^A
60	0	0.012±0.00004 ^B	0.0013±0.00004 ^B	0.0016±0.00002 ^D	0.0010±0.00003 ^B	0.0004±0.00003 ^B	0.0002±0.00002 ^A
75	0	0.012±0.00009 ^B	0.0016±0.00003 ^C	0.0016±0.00003 ^D	0.0016±0.00002 ^E	0.0010±0.00002 ^E	0.0006±0.00002 ^B
90	0	0.012±0.00006 ^B	0.0016±0.00003 ^C	0.0016±0.00002 ^D	0.0012±0.00004 ^C	0.0004±0.00002 ^B	0.0002±0.00002 ^A
105	0	0.012±0.00007 ^B	0.0016±0.00003 ^C	0.0016±0.00003 ^D	0.0016±0.00005 ^E	0.0008±0.00003 ^D	0.0006±0.00002 ^B
120	0	0.012±0.00008 ^B	0.0019±0.00002 ^E	0.0018±0.00003 ^E	0.0016±0.00003 ^E	0.0016±0.00003 ^H	0.0010±0.00002 ^D
135	0	0.012±0.00003 ^B	0.0019±0.00002 ^E	0.0012±0.00003 ^B	0.0012±0.00002 ^C	0.0010±0.00002 ^E	0.0008±0.00002 ^C
150	0	0.012±0.00008 ^B	0.0018±0.00001 ^D	0.0014±0.00004 ^C	0.0012±0.00002 ^C	0.0008±0.00002 ^D	0.0008±0.00002 ^C
165	0	0.012±0.00004 ^B	0.0018±0.00003 ^D	0.0016±0.00002 ^D	0.0012±0.00003 ^C	0.0014±0.00003 ^G	0.0008±0.00002 ^C
180	0	0.012±0.00005 ^B	0.0020±0.00002 ^E	0.0016±0.00002 ^D	0.0012±0.00003 ^C	0.0012±0.00003 ^F	0.0012±0.00002 ^E
195	0	0.011±0.00011 ^C	0.0023±0.00004 ^F	0.0020±0.00001 ^F	0.0014±0.00003 ^D	0.0016±0.00002 ^H	0.0012±0.00002 ^E
210	0	0.010±0.00008 ^C	0.0019±0.00003 ^D	0.0016±0.00003 ^D	0.0012±0.00002 ^C	0.0008±0.00002 ^D	0.0010±0.00002 ^D

Values are represented as Mean ± SD, n=3; ^{A, B} – different superscript column-wise 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 of SFO @ 5%;

Wd: PG spiked with SFO @ 2.5%; We: PG spiked with SFO @ 1%;

4.3.6 Evaluation of the developed rapid color sensor in market ghee samples and validation through RP-TLC method to detect vegetable oils adulteration in ghee

The response time of the paper disc-based color sensor in branded and unbranded ghee samples has been discussed in this section. It is evident from the results (Table 4.27) that observed response time in branded ghee samples coded as C, D, E and F was too less i.e., less than 10 min and it varied from 1- 9 min. It is evident from the discussion in previous sections (4.3.3) that response time of the sensor in antioxidants containing ghee samples was less than 30 min. The results obtained in the study related to branded ghee samples clearly indicated that the samples C, D, E and F were found to have vegetable oils.

Unbranded samples, coded as 1,2,3 and 5, the response time of the developed paper disc-based sensor was in the range of 1- 12 min. The response time obtained in these sample indicated that there could be the presence of vegetable oils in these samples.

It is already established that the developed sensors work in the presence of antioxidants, hence the samples found positive were further checked for their purity using the RP- TLC method. The RP- TLC method works on the presence of β - sitosterol, a sterol of plant origin and is invariably present in the vegetable oils.

Table 4.27 Performance of the developed paper-based disc sensor in branded and unbranded market samples of ghee

Ghee samples	Code	Response time (min)	Inference (+ve/-ve)
Branded	A	246 $\pm$ 10	(-Ve)
	B	240 $\pm$ 8	(-Ve)
	C	9 $\pm$ 1	(+Ve)
	D	9 $\pm$ 1	(+Ve)
	E	1 $\pm$ 0	(+Ve)
	F	1 $\pm$ 0	(+Ve)
Unbranded	1	1 $\pm$ 0	(+Ve)
	2	12 $\pm$ 1	(+Ve)
	3	3 $\pm$ 0.5	(+Ve)
	4	287 $\pm$ 10	(-Ve)
	5	2 $\pm$ 0.5	(+Ve)
	6	302 $\pm$ 12	(-Ve)

Values are represented as Mean $\pm$ SD, n=3

Table 4.28 Comparison of paper- based disc sensor with RP-TLC analysis of branded and Unbranded market ghee samples

Ghee samples	Code	Response time (min)	Disc sensor Inference (+ve/-ve)	RP-TLC method (+ve/-ve)
Branded	A	246±10	(-Ve)	(-Ve)
	B	240±8	(-Ve)	(-Ve)
	C	9±1	(+Ve)	(-Ve)
	D	9±1	(+Ve)	(+Ve)
	E	1±0	(+Ve)	(-Ve)
	F	1±0	(+Ve)	(+Ve)
Unbranded	1	1±0	(+Ve)	(-Ve)
	2	12±1	(+Ve)	(+Ve)
	3	3±0.5	(+Ve)	(+Ve)
	4	287±10	(-Ve)	(-Ve)
	5	2±0.5	(+Ve)	(+Ve)
	6	302±12	(-Ve)	(-Ve)

Values are represented as Mean ± SD, n=3

It is evident from the plate-1 depicting the results of RP- TLC of these samples, that branded ghee samples (D and F) and unbranded samples (2, 3, and 5) showed the presence of a band corresponding to the band of β - sitosterol. This confirmed that the said samples contained vegetable oils and deemed to be adulterated with vegetable oils.

From the above discussion it was found that some of the samples which were found to be positive for vegetable oils on the basis of paper-based disc sensors, showed absence of vegetable oils in RP- TLC analysis. This created a curiosity to find out the possible reason of their positive response to the developed sensor. Hence, these samples were analyzed for their antioxidant profile using the GLC method standardized in the present study. It is evident from the results (Fig 4.34 and Fig 4.35) of GLC analysis of the corresponding positive samples that a peak corresponding to the retention time of TBHQ was appeared. This clearly indicated that there was the possibility of use of antioxidant TBHQ by the manufacturer of these ghee samples, though it is not permissible under FSSR, 2011.

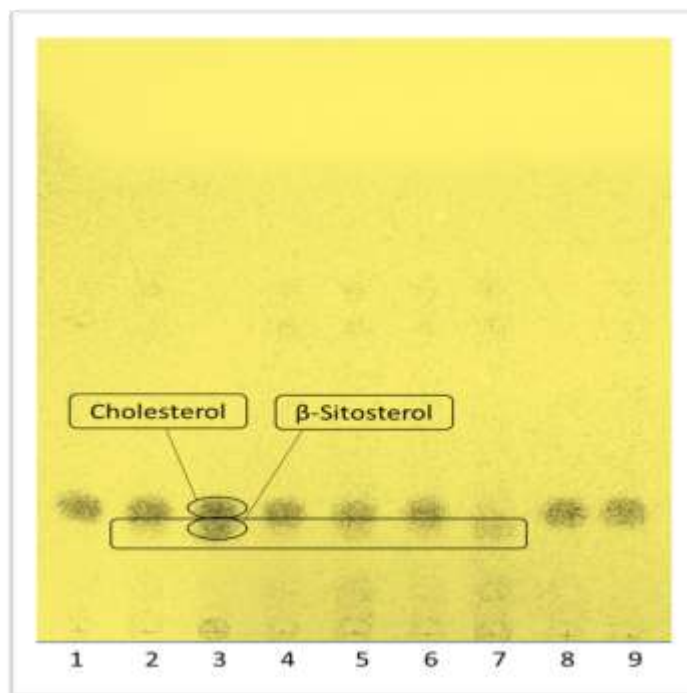


Plate 4.1 Branded and unbranded ghee sample: 1: Buffalo ghee (NDRI); 2: Unbranded Ghee; (2); 3: Standard mixture (cholesterol + β -sitosterol); 4: Unbranded Ghee (3); 5: Unbranded Ghee (5); 6: Branded ghee (D); 7: Branded ghee (F); 8: Cow ghee (NDRI) 9: Cotton seed fed (30% for 60 days) ghee

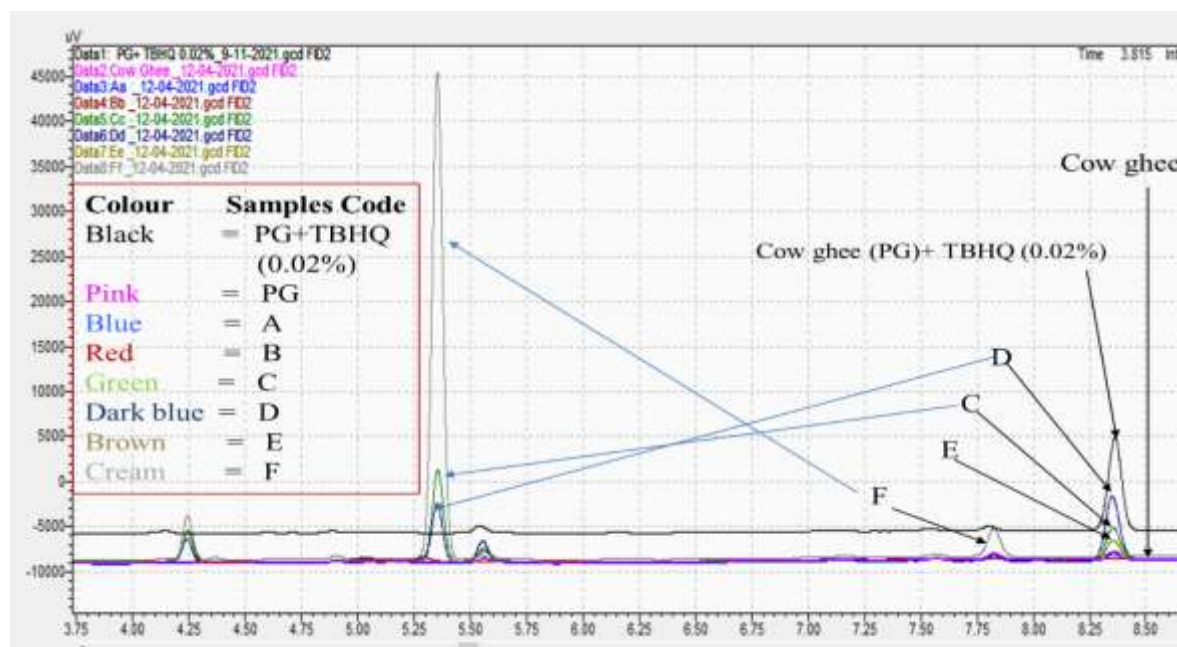


Fig. 4.34 Antioxidant's profile of branded ghee samples

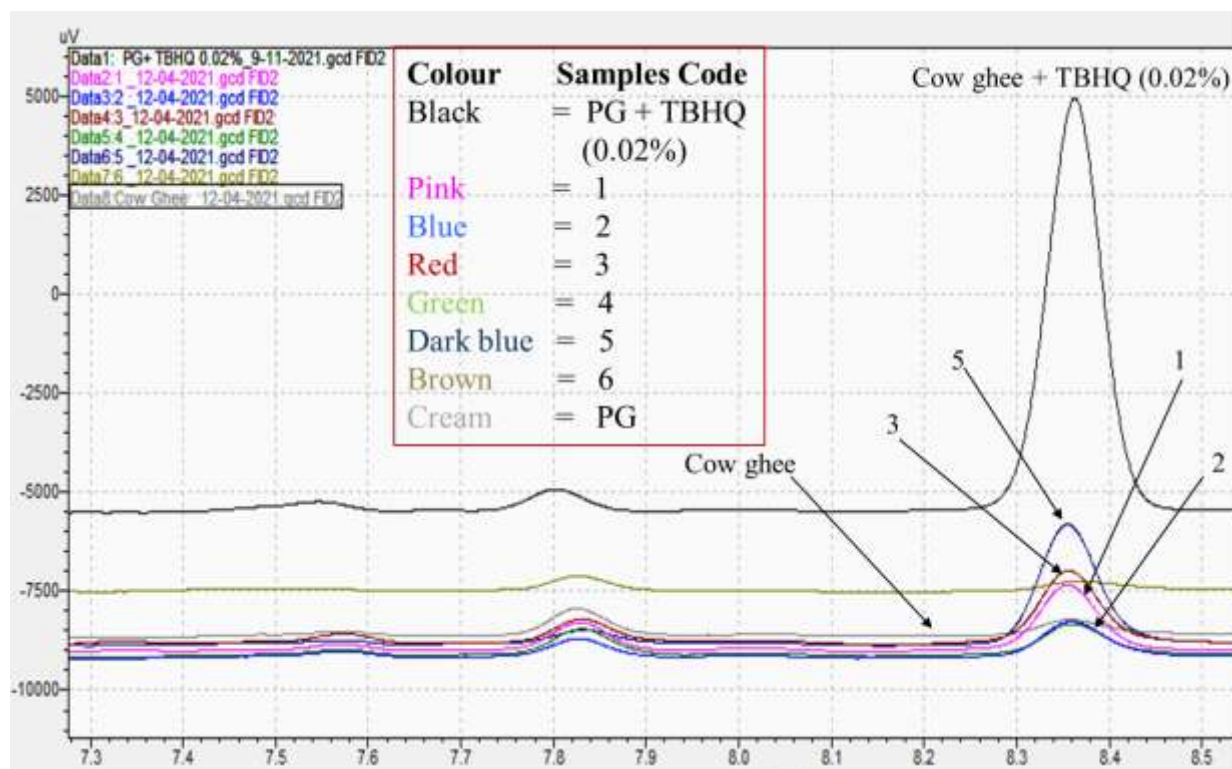


Fig. 4.35 Antioxidant's profile of unbranded ghee samples

CHAPTER - 5

Summary and Conclusions

Summary and conclusions

A systematic work was planned to develop the color sensor based rapid method for detection of vegetable oils adulteration in ghee. Objective first of the present study was the development of color sensor for synthetic antioxidant detection. Objective second was to study the storage stability of developed rapid color based sensor and objective third was evaluation of developed rapid color-based sensor (Tube/Card/Disc) in stored and market ghee samples. In this chapter the important findings of the present study have been summarized as following points:

1. 20 μ L of 4mM DPPH solution were found to be optimum to give a perceptible color change.
2. The optimized level worked efficiently even in the permissible levels of Gallic acid (GA), Ascorbic acid (AA) and a scorbyl palmitate (AsP), maximum permitted levels as per FSSAI 0.02, 0.03 and 0.05%, respectively.
3. 20 μ L of 20% nanoceria (CeO_2) dispersion, as well as impregnated dried paper-based disc did not show color change in the presence of BHA and TBHQ concentrations from (0.02, 0.01, 0.005 and 0.0025) %.
4. 20 μ L of 20% CeO_2 dispersion and CeO_2 impregnated dried paper disc showed color change in GA, AA & AsP.
5. Double extraction was found more effective to extract antioxidants from ghee, as the response time was approximately same as observed in standard solutions of antioxidants.
6. color change in Card sensor after adding antioxidants was not sharp, hence this approach was not perused further.
7. The disc and tube sensors worked well in the presence of synthetic antioxidants as the color change was sharp and perceptible with naked eyes and response time was similar to the liquid dye in standard antioxidants solution.
8. The storage stability of tube sensor was observed for 15 days at ambient temperature (30°C) and 30 days at refrigerated temperature ($4-6^\circ\text{C}$).
9. The storage stability of disc sensor was 30 days at ambient temperature (30°C) and 90 days at refrigerated temperature ($4-6^\circ\text{C}$).
10. The column CP7532(5.0m x 0.53mm (ID) x 0.80 mm (OD), 0.17 μm), retention times of BHA and BHT were observed to be same (1.9 min) and therefore, this column and conditions were not used in the further study.

11. The column SPB-5(30m x 0.53 mm x 1.5µm) could resolve the different synthetic antioxidant as the retention times were observed for BHA, BHT and TBHQ antioxidants were 7.45, 8.0 and 8.35 min, respectively.
12. GLC method was found to be nonlinear below 0.0025% concentration of the antioxidants. Therefore, the indirect method i.e., extraction of antioxidants before GLC analysis was found suitable. GLC method was linear for both BHA and TBHQ at or above 0.0025% concentration of antioxidant.
13. In case of direct method i.e., GLC of ghee samples directly without extracting the antioxidants, the method was found to be linear above 0.02% of antioxidant concentration.
14. Recovery in case of indirect method (GLC after extracting the antioxidants), was found to be 97.73±4.98 and 97.59±5.38 for TBHQ and BHA, respectively.
Peroxide value of all the samples increased during the storage period and this increase was observed in the following order: PO >SFO> PO spiked PG> SFO spiked PG>PG>BHA spiked PG> TBHQ spiked PG.
15. TBA value of all the samples increased during the storage period and this increase was observed in the following order: SFO> SFO spiked PG> PG> PO spiked PG> PO>BHA spiked PG> TBHQ spiked PG.
16. Using the developed paper-based disc sensor, a distinct color change was seen in case of cow ghee samples spiked with palm oil and sunflower oil @ 1%.
17. It was observed that in case of ghee samples spiked with PO @1% and subsequently stored, the response time of paper-based disc sensor came close to 240 min after 5 months of storage. However, in case of cow ghee samples spiked with sunflower oil and subsequently stored, the response time of the paper-based disc sensor was very much below the time of 246 min even after 9 months of storage. It was found that in stored samples of ghee it is possible to detect the added vegetable oils at a level of about 5%, because storage lead to a significant increase in the response time of the sensor, especially in ghee samples spiked with lower level of vegetable oils.
18. Developed disc sensors were evaluated in market ghee samples (6 branded and 6 unbranded), it was found that 4 samples out of the 6 in each case showed the response time in the range of 1- 12 min, which was well below the time of 246 min indicating the presence of antioxidants.

19. GLC analysis of the corresponding positive samples also showed the presence of a peak corresponding to the retention time of TBHQ.
20. Reversed- phase thin layer chromatography (RP-TLC) analysis of branded samples showed the presence of β - sitosterol in 2 samples, whereas in case of unbranded samples, β - sitosterol appeared in 3 samples. This indicated that not only the vegetable oils but the synthetic antioxidants are also being added in ghee samples available in the market.

The present study resulted into to the development of a paper-based disc sensor, which was able to detect the adulteration of cow ghee with vegetable oils at the level of 1% and more. In case of stored samples, it was possible to detect the presence of vegetable oils at the level of 5% and above. These developed sensors can be stored for 30 days at ambient temperature (30°C) and 90 days at refrigerated temperature (4-6°C). The developed sensors will cater to the difficulty in procuring the dyes and making the solutions at milk collection centers. Study also showed that developed sensors are rapid and can be used at the chilling center or milk collection centers, which will enable the dairy processors to counter the problem of milk fat adulteration with vegetable oils. All the experiments were conducted in cow ghee samples. Hence, the sensor developed in the present work was checked for its effectiveness in detecting the cow ghee adulteration with vegetable oils. To extrapolate these results in buffalo ghee and mixed ghee samples, there is a need to validate the developed sensor to check its efficacy in buffalo ghee or mixed ghee (cow and buffalo ghee) spiked with vegetable oils.

CHAPTER - 6

Bibliography

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