EFFICACY OF BAUDOUIN AND SESAMIN TESTS TO DETECT VANASPATI/ HYDROGENATED VEGETABLE OIL IN GHEE DURING STORAGE

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

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY

BY

MACWAN KAUSHIKKUMAR ARVINDBHAI
B. Tech (DT)

DAIRY CHEMISTRY DIVISION
ICAR-NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
KARNAL – 132001 (HARYANA), INDIA

2016

Regn. No. 14-M-DC-07

EFFICACY OF BAUDOUIN AND SESAMIN TESTS TO DETECT VANASPATI/ HYDROGENATED VEGETABLE OIL IN GHEE DURING STORAGE

BY
MACWAN KAUSHIKKUMAR ARVINDBHAI

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

MASTER OF TECHNOLOGY

IN

DAIRY CHEMISTRY

Approved by

28/7/2016
EXTERNAL EXAMINER

23/7
Dr. SUMIT ARORA
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

MEMBERS, ADVISORY COMMITTEE

Dr. VIVEK SHARMA
PRINCIPAL SCIENTIST, DC DIVISION — 23.7.16

Dr. RAMAN SETH
PRINCIPAL SCIENTIST, DC DIVISION

Dr. A.K. SINGH
PRINCIPAL SCIENTIST, DT DIVISION

Dr. KAUSHIK KHAMRUI
SENIOR SCIENTIST, DT DIVISION — 25/7/16

DAIRY CEMISTRY DIVISION
NATIONAL DAIRY RESEARCH INSTITUTE
(DEEMED UNIVERSITY)
(Indian Council of Agricultural Research)
KARNAL -132001 (HARYANA), INDIA

Dr. SUMIT ARORA
PRINCIPAL SCIENTIST

CERTIFICATE

This is to certify that the thesis entitled "**EFFICACY OF BAUDOUIN AND SESAMIN TESTS TO DETECT VANASPATI/ HYDROGENATED VEGETABLE OIL IN GHEE DURING STORAGE"** submitted by **Mr. MACWAN KAUSHIKKUMAR ARVINDBHAI** in partial fulfillment of the requirement for award of the degree of **MASTER OF TECHNOLOGY** in **DAIRY CHEMISTRY** of the **ICAR-NATIONAL DAIRY RESEARCH INSTITUTE (Deemed University)**, KARNAL (HARYANA) 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: 7/7/2016

(Dr. SUMIT ARORA)
MAJOR ADVISOR AND CHAIRMAN
(GUIDE)

Dedicated to

My Parents

ACKNOWLEDGEMENT

It gives me immense pleasure to express my heartiest gratitude to my guide Dr. Sumit Arora, Principal Scientist, Dairy Chemistry Division, NDRI, Karnal (Haryana) for his valuable guidance, continuous encouragement and moral support during the entire period of the study. I am extremely indebted to my guide for providing necessary resources at exact time to accomplish my research work. His unflinching courage and conviction will always inspire me, and I hope to continue to work with his noble thoughts. I am thankful to the Almighty for giving me a mentor like him.

Also, I have been fortunate to receive the guidance of Dr. Vivek Sharma, Principal Scientist, Dairy Chemistry Division and my advisory committee member. Had it not been for his driving spirit, right from the conception of the project to completion of the work, this project could not have been effectively carried out. I feel proud to be a researcher under his inspiring guidance. Under his guidance I successfully overcame many difficulties and learned a lot I am highly obliged to him for the unconditional support offered throughout the research work. Without his altruistic help and keen interest, it would not have been possible to bring the thesis to this form.

I would like to express my myriads of thanks to members of my advisory committee, Dr. Raman Seth, Dr. A.K.Singh and Dr. Kaushik Khamrui for their keen interest, valuable suggestions and cooperation. I am deeply grateful to them for the long discussions that helped me sort out my research work problems.

I express my deep sense of gratitude to Dr. A.K. Srivastava, Director NDRI, Karnal, for providing necessary facilities to carry out the present investigation. Financial assistance in the form of Junior Research Fellowship from ICAR is duly acknowledge that help me to carry out the research work smoothly.

A lot of thanks are due to all the Scientists, Technical Officers and Staff of the Dairy Chemistry Division, for their sagacious guidance, continuous encouragement and intellectual stimulation throughout the tenure of my study. I am indebted to the valuable help rendered by P.C. Singh sir, Ingle ji, Jagat ji, Kulwinder ji and Priya mam for their kind cooperation. I shall cherish my association with dear juniors Nakul, Mitul and Sachin , and my best wishes are with them.

There are no words to convey my deep gratitude and respect for my one of best senior Apurva mam who helped me throughout my research work and thanks to Chitra mam, Syama mam, Sonika mam, Shilpa Mam, Anuradha mam, Kashyap sir, Ravi sir and Prince sir for their cooperation, valuable guidance and sustained encouragement during the course of study. Thanks for being with me and providing great support. My heartiest thanks my dear friends Rakesh, Gautam, Hitesh, Sonu, Amol, Harisha, Sowmya and my other batchmates who have been a constant source of love, concern, support and strengthened me throughout the course of M.Tech programme. Thanks for being always with me and the memorable and joyful time spent together will always be remembered. I have been lucky to have Gagan from DM Division who ensured that I never feel homesick during my stay in Karnal and always was there as moral boost. I would like to elicit the gesture of indebtedness to Anupama mam and Alka mam for having helped me affectionately from time to time and the guidance they offered throughout my stay in the salutary Lab no. 228.

More words cannot express my unbound regards to Angel, for her insightful interactions, friendly approaches and sacrifices.

Last but not the least, none of this would have been possible without the unconditional love and patience of my family. My Parents to whom this dissertation is dedicated to. I would like to express my heart-felt gratitude to my family who has always stood firmly and encouraged me throughout this journey.

I thank ***Almighty God*** *for blessing me with everything I can ever ask for.*

Dated: (*Kaushik Macwan*)

Contents

List of Tables

List of Figures

ABBREVIATIONS

%	Percent
μg	Microgram
μl	Microlitre
g	Gram
mg	Milligram
min	Minutes
ml	Millilitre
N	Normal
ng	Nanogram
nm	Nanometer
^{o}C	Degree Celsius
ppm	Parts Per Million
v/v	Volume By Volume
w/v	Weight By Volume
w/w	Weight By Weight
AGMARK	Agricultural Marking
AOCS	American Oil Chemists' Society
AR	Analytical Reagent
BHA	Butylated Hydroxy Anisole
BHT	Butylated Hydroxy Toluene
BIS	Bureau of Indian Standards
CDA	Conjugated Dienoic Acid
Codex	Codex Alimentarius Commission
FSSAI	Food Safety And Standards Authority Of India
GC	Gas Chromatography
GC/MS	Gas Chromatography/ Mass Spectrometry
HCl	Hydrochloric Acid
LDPE	Low Density Polyethylene
HPLC	High Performance Liquid Chromatography
ML	Methyl Linoleate
NDRI	National Dairy Research Institute
PAV	*p*-Anisidine Value

PDA	Photodiode Array Detector
PFA	Prevention of Food Adulteration
PG	Propyl Gallate
PHVO	Partially Hydrogenated Vegetable Oil
PV	Polenske Value
RM	Reichert Meissl
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RT	Retention Time
USM	Unsaponifiable Matter
UV	Ultraviolet

ABSTRACT

Ghee is the most widely consumed Indian dairy product which holds an important place in Indian diet. It is prepared from cow or buffalo milk or combination thereof. Unfortunately, the producers or the middle-men involved in the ghee trade, in their greed to earn more money, tend to adulterate ghee with cheaper oils and fats e.g. vegetable oils, animal body fats, hydrogenated fats, and sometimes even the non-edible mineral oils, especially during lean season. In recent years, the problem of adulteration has assumed a very serious dimension. Ghee adulterated with vanaspati containing sesame oil can be easily detected using Baudouin test, where the appearance of red coloured complex indicates a positive test. A colour based sesamin test for the detection of sesame oil in vegetable oil has been approved by Codex (1999), however this test was not performed in case of ghee. In the present study, sesamin test was optimized accordingly to detect adulteration of ghee with vanaspati (containing sesame oil) which gave positive results for 10% and above adulterated samples whereas, detection at lower levels of adulteration was not possible. No adverse changes were observed in Sesamin and Baudouin test results throughout the storage period (37ºC for 8 months). It was also observed at packaging material had no adverse effect on Sesamin and Baudouin test. Sesamin and sesamolin were detected on TLC plates for ghee samples adulterated at 5, 10, 20 and 30% during storage (37ºC/8 months and 80ºC/10 days). Detection of sesamin and sesamolin in ghee was possible at 1, 5, 10, 20 and 30 % adulteration using HPLC. Results of HPLC revealed that sesamin was more stable than sesamolin. It was also observed that minimum degradation of sesamin and sesamolin occurred in samples containing BHA in comparison to samples without BHA. During storage of adulterated ghee sample at 80ºC for 10 days, no change was observed in Baudouin test but Sesamin test gave doubtful result for 10% adulterated sample (without BHA), whereas for 10% adulterated sample (with BHA) results were not affected.

सारांश

घी एक सबसे व्यापक तौर पर उपभोग करने वाला भारतीय दुग्ध उत्पाद है जिसने भारतीय आहार में महत्वपूर्ण पकड़ बनाई है| यह गाय अथवा भैंस के दूध अथवा दोनों के मिश्रण से बनाया जाता है| दुर्भाग्य पूर्वक, उत्पादक तथा दलाल ज्यादा पैसे कमाने के लालच में, घी में सस्ते तेल जैसे कि वनस्पति तेल, पशु शरीर की चर्बी, हाईड्रोजनी कृत वसा, गैर खाद्य खनिज तेलों की मिलावट करते है| हाल ही में मिलावट एक बहुत गम्भीर समस्या बन गई है| घी में वनस्पति तेल की मिलावट की जाँच तिल के तेल की मात्रा को बाऊडोवीन परिक्षण के द्वारा परख कर की जा सकती है जिसमे लाल रंग मिश्र इस परिक्षण के होने का संकेतक है| रंग पर आधारित सीसामिन परिक्षण, वनस्पति तेल में तिल के तेल को जाँच करने के लिए बनाया गया है जो कोडेक्स द्वारा मान्यता प्राप्त है, हालाकि यह परिक्षण घी में नहीं किया गया था | वर्तमान अध्ययन में घी में वनस्पति की जाँच करने के लिए सीसामिन परिक्षण को अनुकूलित किया गया जिसमे १०% एवं उससे ऊपर की मिलावटी घी में पोजीटिव नतीजे मिले जबकि निम्न स्तर की मिलावट में यह मुमकिन नहीं था| भंडारण अवधि (३७°C /८ महीने) के दौरान सीसामिन और बाऊडोवीन परिक्षण के नतीजों में कोई भी विपरीत परिवर्तन नहीं पाया गया| पैकेजिंग सामग्री का सीसामिन तथा सिसामोलिन में कोई भी विपरीत परिवर्तन नहीं पाया गया| भण्डारण अवधि के दौरान (३७°C/८ महीने तथा ८०°C/१० दिनों) मिलावटी घी (५, १०, २०, ३०%) में सीसामिन तथा सिसामोलिन की जाँच पतली परत क्रोमैटोग्राफी द्वारा की गई | सीसामिन तथा सिसामोलिन की जाँच मिलावटी घी (१, ५, १०, २०, ३०%) में एच पी एल सी द्वारा किया गया| एच पी एल सी नतीजे यह प्रकाशित करते है कि सिसामोलिन कि तुलना में सीसामिन ज्यादा स्थिर था| सीसामिन तथा सिसामोलिन की अपकर्षता बी एच ऐ विहीन नमूने की तुलना में बी एच ऐ युक्त नमूने में न्यूनतम थी| भंडारण अवधि के दौरान मिलावटी घी में बाऊडोवीन परिक्षण में कोई परिवर्तन नहीं आया परन्तु १०% मिलावटी नमूने (बी एच ऐ विहीन) में संदेह परिणाम आये जबकि १०% मिलावटी नमूने (बीएचऐ युक्त) में कोई अंतर नहीं दिखाई दिया|

CHAPTER –1

Introduction

INTRODUCTION

Ghee is clarified milk fat and is widely consumed in the Indian sub-continent since time immemorial. Its organoleptic attributes, nutritional value and functional properties make it suitable for numerous food applications. Ghee is prepared from cow or buffalo milk or combination thereof. As a human food, ghee has been considered immensely superior to other fats, mainly because of the presence of characteristic short chain fatty acids which are responsible for its better digestibility and anti-cancer property. Apart from having rich and pleasant sensory attributes, ghee is a carrier of four fat-soluble vitamins viz., A, D, E, K and essential fatty-acids such as linolenic acid and arachidonic acid. Ghee blends with the food ingredients without losing its medicinal value. Additionally, it's nutritional and health benefits are touted as ideal for people of all age group. Even in Ayurvedic and Unani systems of medicine, ghee is generally used as the base for culinary purposes, it is the only animal fat universally acceptable by both vegetarian and non-vegetarian populations.

During the period of short supply and more demand, fraudulent traders adulterate milk fat for maximizing profit by addition of cheaper oils and fats of vegetable and animal origin. In recent years, the problem of adulteration has assumed a very serious dimension. Such a situation has tarnished the image of dairy industry, not only in India, but abroad also. 50% of the ghee samples collected from local markets of Coimbatore was found to be of substandard quality (Anon, 2014). Times of India also reported that a ghee making unit had been purchasing ghee in large quantities, adulterating and repackaging it under various brands in Vijayawada (Anon., 2015a). Ghee samples from 14 dairy units in Vijayawada were found to be adulterated, their licenses were cancelled and a penalty of Rs.26.80 lakh was imposed on them (Anon., 2015b). Several reports have appeared in the newspapers indicating that rampant malpractices of ghee adulteration are shooting up day by day on a large scale in almost all parts of the country.

In order to prevent the adulteration with Vanaspati or Partially Hydrogenated Vegetable Oil (PHVO), Government of India formulated Vegetable Oil Products Control Order in 1947. According to this order, addition of 5 percent

sesame oil is compulsory in vanaspati. Presence of lignans like sesamin and sesamolin in sesame oil can be used as tracer. Ghee adulterated with vanaspati containing sesame oil can be easily detected using Baudouin test, where appearance of red coloured complex indicates a positive test. Similarly, a colour based Sesamin test for the detection of sesame oil in vegetable oil has been approved by Codex (1999), in which development of bluish green colour indicates the presence of sesamin. Sesamin is considered to be more stable compound as compared to sesamolin. However, this test has not been studied in case of ghee, where in it could be the better alternative/ or additional test to confirm the vanaspati in ghee.

According to the existing procedure for collection and analysis of food samples approved by FSSAI, the food analyst has to send the report of analysis within 14 days from receipt of the sample. The major drawback in this procedure is that during the above given period, if the deterioration of adulterant occurs, the test results will be negative. Baudouin and Sesamin test are based on the presence of the sesame lignans like sesamolin and sesamin, respectively. These sesame lignans tends to degrade with time which may cause the false negative results which can mislead and encourage adulteration and lead to acquittal of fraudulent traders. No work has been reported in literature on the evaluation of Baudouin and Sesamin test on the stored ghee samples. Therefore, the present research has been undertaken in order to assess the efficacy of Baudouin and Sesamin test to detect vanaspati in stored ghee samples with the following objectives:

1. Profiling of tracer components (Sesamin and Sesamolin) using chromatographic procedure and correlation with Baudouin and Sesamin test
2. To examine the efficacy of Baudouin and Sesamin test in vanaspati adulterated ghee with and without addition of BHA during storage

CHAPTER –2

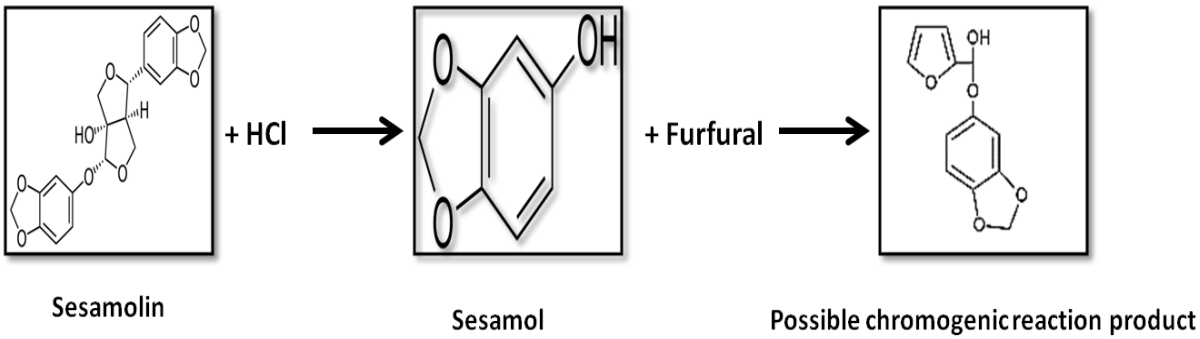
Review of Literature

REVIEW OF LITERATURE

In India, milk fat is mostly consumed in the form of ghee (clarified butterfat). It is considered to be nutritionally superior over other fats since it is a rich source of fat soluble vitamins, essential fatty acids and possess pleasant sensory attributes. The rich flavour of milk fat cannot be easily duplicated by other fats. In India, critical situation arises when the supply of milk and ghee falls short of the demand. Unscrupulous people in the trade take undue advantage of such situations and indulge in the malpractice of adulteration of milk and ghee with different types of adulterants. Milk fat being the costliest constituent of milk is adulterated with cheaper fats such as vegetable oils, animal body fats, hydrogenated fats, interesterified fats, etc for the manufacture of ghee.

A few surveys have been carried out all over India to ascertain the quality of ghee sold in market. Subrahmanyam *et al.* (1952) collected large number of samples from Bangalore and Mysore markets and reported that one-third of the samples did not contain ghee at all and one-half of the total samples were grossly adulterated. Sharma and Zariwala (1978) carried out quality evaluation of ghee samples from Bombay city and found that 19% failed with respect to moisture and 18% failed with respect to RM and PV values in PFA and AGMARK standards. Baudouin test was found positive in 20% of the samples. Rao *et al*. (2004) analysed the ghee samples from organized and unorganized market of Hyderabad and showed that 35 % of the samples from the local market gave positive Baudouin test.

In recent years, the problem of adulteration has assumed a very serious dimension. Such a situation has tarnished the image of the Indian dairy industry. In order to ensure a genuine product to the consumer, the Government of India formulated Vegetable Oil Products Control Order in 1947. According to this order, addition of 5 % sesame oil is compulsory in vanaspati (hydrogenated fat). Presence of lignans like sesamin and sesamolin in sesame oil can be used as tracer for its presence in vanaspati. Ghee adulterated with vanaspati containing sesame oil can be easily detected using Baudouin test, where appearance of red coloured complex indicates a positive test. The chemical reaction is as follows:



(Lee *et al.* 2013)

Figure 2.1 Possible chromogenic mechanism to detect sesame oil

Extensive survey of the literature revealed that several methods have been developed to detect the adulteration in ghee. These methods were mostly based on chemical parameters like fatty acid composition and the physico-chemical constants but very few attempts have been made to detect the adulteration on the basis of minor constituents such as sesame lignans (sesamin and sesamolin) which comes through adulteration of vanaspati. This review delineates the current status of our knowledge with regard to the various methods commonly used for the detection of adulteration of milk fat with vanaspati.

2.1 Sesame oil

Sesame oil contains high level of unsaturated fatty acids (more than 80% of total fatty acids) although it is highly resistant to oxidative deterioration as compared with other edible vegetable oils. The superior oxidative stability is not only attributed to the presence of tocopherols, but it is mainly associated with the unique group of compounds lignans. Two types of lignan compounds exist in sesame seeds, the oil soluble lignans and the water soluble lignan glycosides. In raw sesame seed, sesamin and sesamolin are the two major lignans. Sesamin has been found in other plants, whereas sesamolin is characteristic of sesame and has not been found in plants other than *Sesamum*. Structures of sesame lignans are shown below:

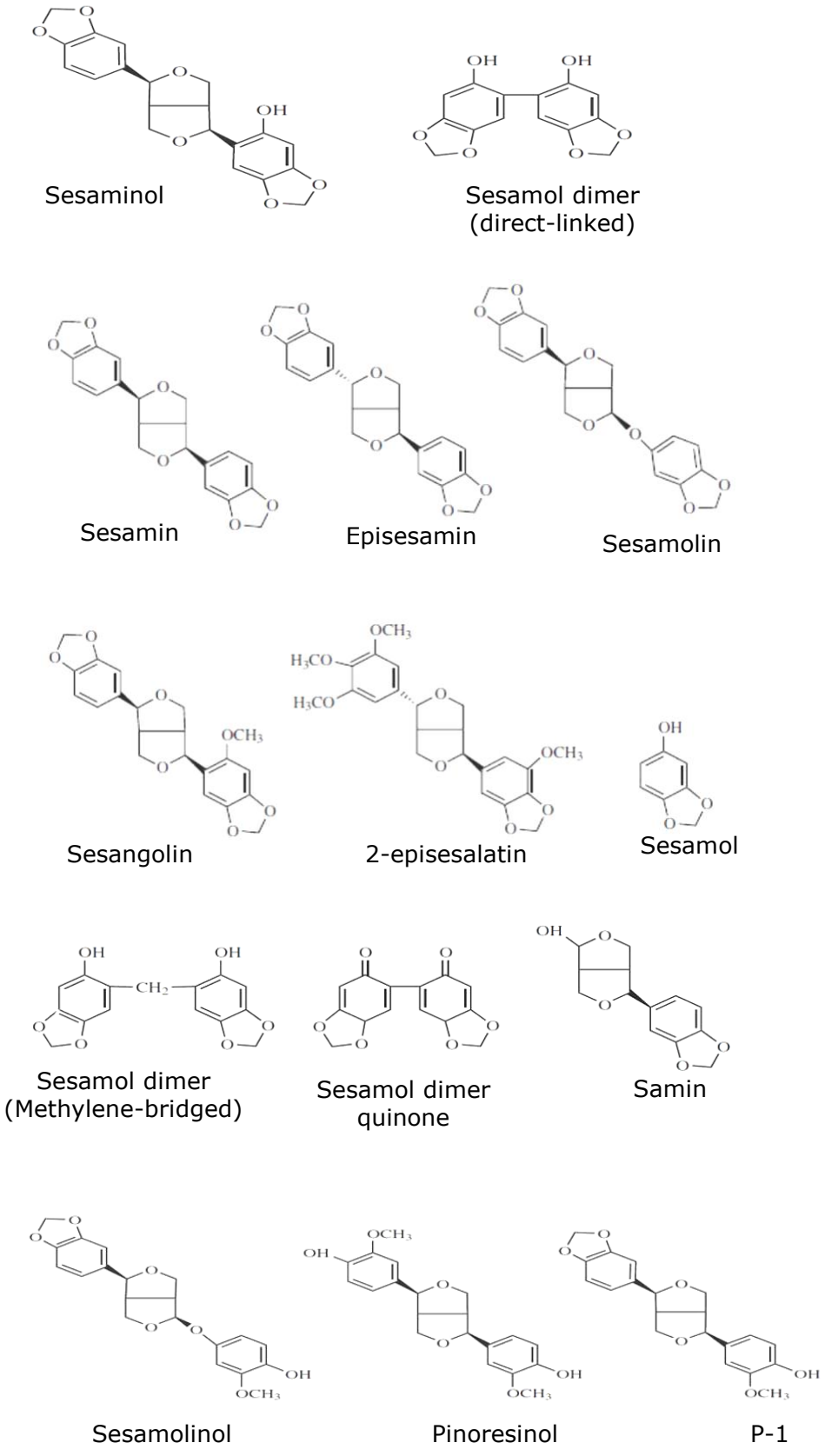


Figure 2.2 Structure of sesame lignans (Hwang, 2005)

Table 2.1 Contents of sesame lignans in unroasted sesame oil during industrial refining process (mg/100 g oil)

Refining stage	Sesamin	Sesamolin	Sesamol
Crude sesame oil	813.3	510	4.3
Alkaline refining	730.6	458	2.5
Warm water washing	677.8	424.8	0.7
Bleaching	375.5	0	46.3
Deodorizing	258.3	0	1.7

2.2 Detection of sesame lignans by various techniques

Kamal-Eldin *et al.* (1994) carried out comparison of different chromatographic methods for analysis of lignans in seed oil from four *Sesamum* species. Thin Layer Chromatography (TLC), Gas Chromatography (GC), Gas Chromatography/ Mass Spectrometry (GC/MS) and Reversed Phase High Performance Liquid Chromatography (RP-HPLC), methods were compared for their ability to separate the different lignans present in four *Sesamum* species, *viz., S. indicum* Linn., *S. alatum* Thonn*., S. radiatum*and *S. angustifolium.* Two dimensional TLC was found to be a valuable qualitative technique and one-dimensional TLC was useful for preparative purposes. GC/MS technique confirmed the identity of the lignans present in sesame seed oil. RP-HPLC was unable to separate sesamolin and sesangolin but a normal-phase silica column provided satisfactory separation for these two lignans.

Amarowicz *et al. (*2001) purified sesamin and sesamolin using semipreparative RP-18 HPLC. Sesamin and sesamolin from sesame oil was separated by classical column chromatography using alumina and petroleum ether to collect furofuran lignans. Semipreparative RP-18 HPLC was used for further separation of furofuran lignans with high purity (>99%).GC/MS investigation confirmed that isolated compounds were sesamin and sesamolin.

Li *et al.* (2002) carried out characterization and analysis of *Semen Causcutae* by capillary gas chromatography and GC/MS. The pharmaceutically active component (+) sesamin was identified and determined in this plant for the

first time. Several n-alkanes, (+) sesamin, and some compounds were identified by GC/MS and GC retention index and they were chosen to characterize different samples of *Semen Cuscutae*. Fingerprints of the extracts of *Semen Cuscutae* from six sources were also obtained.

Eight tetrahydrofurofuran nucleus lignans such as syringaresinol, *epi*-syringaresinol, lirioresinol-B dimethylether, xanthoxylol, phillygenin, fargesin, sesamin and asarinin were separated on a C18 column in the RP-HPLC. The central nucleus (tetrahydrofurofuran) on which these lignans are built, forms *trans*-form and *cis*-form stereoisomers presented satisfactorily through Newman projections. Upon examination, the relatively strain less *cis*-form was found more probable than the puckered and very strained *trans*-form. All tetrahydrofurofuran nucleus lignans elucidated have been derived from the *cis*-form. In the case of diastereomers such as sesamin and asarinin, Newman projections indicated that the diagonal substituents on the structure of sesamin were on the same side of the tetrahydrofurofuran nucleus and were bent toward each other. As a result, the surface of the molecule that is in contact with the stationary phase during elution is thought to be smaller than that for those on the opposite side (i.e., asarinin). As a result of less retention, a shorter elution time can be expected for sesamin (Wang *et al*. 2003).

Kim *et al*. (2006) also determined sesamin and sesamolin in sesame (Sesamum *indicum L*.) seeds using UV spectrophotometer and HPLC. The sesamin and sesamolin contents were determined by HPLC analysis of methanol extract and their total lignan content was compared with UV-Vis spectrophotometric analysis. They concluded that the UV values from both the extracts were 3.8-4.7 times higher than those of HPLC values. Lignan content was overestimated by UV method because total compounds in the mixture of solution were quantified by absorbing at the same ultraviolet wavelength as in HPLC method.

Adegbola *et al.* (2008) studied spray reagents for the visualization and detection of sesame oil unsaponifiables on Thin Layer Chromatograms. They used anisaldehyde and crotonaldehyde as spray reagents for the visualization of major components of sesame oil unsaponifiable matter. Colours produced using anisaldehyde were shades of the spectrum, which are characteristic of the

lignans and sterols in the Sesamum *indicum*. It had relatively higher sensitivity and selectivity. However, crotonaldehyde could detect four of the eleven unsaponifiable matters in relatively more stable and bright colour spots.

Table 2. Colour reaction of sesamin and sesamolin with crotonaldehde or anisaldehyde

Compounds	Colour of spots and detection limits (µg)		Retention factor
	Crotonaldehyde	Anisaldehyde	
Sesamin	Brown (0.09)	Purple → yellow(0.15)	0.51
Sesamolin	Brown (0.1)	Purple → blue(0.4)	0.58

Nutraceutical aspects of sesame oil are well reported but an efficient process for commercial production has not yet been reported. Sesame oil was subjected to sequential extraction with methanol under selected conditions of temperature (70ºC), time (100 min) and solvent: oil ratio (1:1). Under the optimised conditions, the yields of methanolic extract concentrate and residual oil were 10.09 ± 1.0 g and 89.2 ± 1.0 g respectively. On HPLC analysis, the methanol concentrate showed a total lignan content of 9.32 ± 0.19% (6.54 ± 0.12% sesamin and 2.78 ± 0.31% sesamolin). The process describes a simple and less cumbersome procedure which included extraction and crystallization techniques. The additional saponification step makes the process most economical and effective for producing lignans with high yield and purity (Reshma *et al.* 2010).

The HPLC analysis indicated that the main sesame lignans in seeds and oils were sesamin and sesamolin. In addition, sesame oil samples also contained some lignan glucosides, e.g. sesaminol, diglucoside and triglucoside. Sesamin and sesamolin are partially insoluble in water. Therefore, 80% methanol was used to extract these compounds from sesame seeds and oils. The distribution plot of sesamin and sesamolin content in seeds showed that the mean values of sesamin and sesamolin were 1.55 mg/g and 0.62 mg/g, respectively. In commercial sesame oils, the ranges of sesamin, sesamolin and tocopherol contents were 0.93–2.89 mg/g, 0.30–0.74 mg/g and 304– 647 µg/g of oil,

respectively. The detection limits of the method were 0.04 µg/ml for sesamin and 0.1 µg/ml for sesamolin (Rangkadilok *et al. 2010*).

Wang *et al.* (2012) analysed sesamin and sesamolin contents by HPLC and revealed that the core sesame germplasm had broad range variation in sesamin and sesamolin from 2.49 to 18.01 mg/g with average of 8.54 mg/g. Sesamin content in white seed coat colour were significantly higher than brown, yellow and black seed coat colour.

Schwertner and Rios, *(*2012) analysed sesamin, asarinin, and sesamolin on HPLC using reversed phase C18 columns with Photodiode and Fluorescent Detector and by GC/MS. They reported that analytical recoveries of sesamin, asarinin, and sesamolin from sesame oil were 92–94 % with two methanol extractions. The limit of quantitation with the fluorescent detector was 0.1 ng compared to 0.1 µg with the PDA detector. The C18 columns showed more effective resolution of sesamin, asarinin, and sesamolin and shorter retention times than C8 columns. Methanol: water (70:30, v/v) was also compared to methanol: water (80:20, v/v) as potential mobile phases. Methanol: water (70:30, v/v) was selected as the HPLC mobile phase since it resolved sesamin, asarinin, and sesamolin more effectively than methanol: water (80:20, v/v).

Liang *et al.* (2012) separated sesamin and sesamolin by a supercritical fluid simulated moving bed. Sesame oil was first obtained from the sesame seeds by supercritical carbon dioxide extraction. The lignans in the oil were enriched and precipitated by supercritical fluid fractionation technology (SFF) and the crude lignans are then separated by supercritical fluid-simulated moving bed chromatography (SF-SMB) to obtain pure sesamin and sesamolin

Arakawa *et al.* (2012) developed a fluorimetric assay of horseradish peroxidase using sesamol as substrate. This horseradish peroxidase assay was highly sensitive. Sesamol was reacted enzymatically in the presence of hydrogen peroxide to produce dimeric sesamol. The dimer was fluorescent and could be detected sensitively at ex.347nm and em.427nm. The measurable range of horseradish peroxidase was 1.0×10^{-18} to 1.0×10^{-15} mol/assay, with a detection limit of 1.0×10^{-18} mol/assay. Sesamol was extracted from sesame oil and could be purchased as an inexpensive general reagent.

Haribabu *et al*. (2014) developed a simple, rapid and accurate HPTLC-MS method for the simultaneous quantification of asaranin and sesamin, the two epimeric furofuran lignans in *Piper chaba* (Piperaceae). Separation of asaranin and sesamin was achieved on 20×10 cm pre-coated silica gel 60 F254S HPTLC plates with ethyl acetate: hexane (30:70) as mobile phase. Detection and quantification were performed densitometrically at λ_{max} 295 nm. The peak identity was confirmed by electron spray ionization mass spectra, which showed the $[M + Na]^+$ ions for both asaranin and sesamin at *m/z* 377. The method was validated in terms of limit of detection, limit of quantification, selectivity, linearity, precision and accuracy. The method was found to be reproducible and convenient for quantitative analysis of asaranin and sesamin in different *Piper chaba* fruit extracts.

Bhatnagar *et al.* (2015) developed a spectrophotometric method for the rapid estimation of total lignans present in sesame oil based on ultraviolet (UV) absorption and specific extinction value of sesamin and sesamol. The method was simple, cost effective, rapid and did not require sophisticated equipments etc. The specific extinction value obtained at characteristic UV absorbance of reference standards sesamin (λ_{max} at 288 nm) and sesamol (λ_{max} at 297 nm) was used to calculate the amount of lignans in sesame oil. They suggested that the UV method could be used for the rapid and near accurate estimation.

Dar *et al*. (2015) reviewed on successful isolation and characterization of the two lignans from commercial sesame oil. A mixture of the oil with acetone in the ratio of 1:8 left for freezing at −80ºC for overnight. These treatments led to precipitation of triglycerides which were then separated by vacuum filtration through a 0.45 µm nylon membrane at 4ºC. The filtrate was subjected to rotary evaporation to yield yellow oil. When yellow oil was subjected to a similar treatment but with isooctane at 4ºC yielded colourless crystalline product. TLC followed by HPLC revealed that the crystalline product was a mixture of 88% sesamin and 12% sesamolin. The isolated lignan mixture was subjected to semi-preparative HPLC and TLC to result in successful separation of sesamin and sesamolin in two independent fractions. Purity of the compounds thus isolated was confirmed by TLC and Liquid chromatography–mass spectrometry. Structure

detail of the isolates was confirmed by Nuclear magnetic resonance spectroscopy.

2.3 Detection of sesame oil based on colour reaction

2.3.1 Soltsein test

It involves the use of Bettendorf's reagent (solution of stannous chloride in concentrated hydrochloric acid) for the detection of sesame oil in olive oil. Appearance of rose to violet colour indicates the presence of sesame oil. It was later elaborated that it is more sensitive than the Baudouin or Villavecchia test (Budowski and Markley, 1951).

2.3.2 Bishop test

According to the Bishop-Kreis test, if oil is exposed to sunlight for a few days and then treated with concentrated hydrochloric acid, a green colour is observed in the presence of sesame oil. This test could be used for the detection of rancid vegetable oils. The Baudouin and Bishop tests can be used to detect the presence of sesamolin in sesame oil (Budowski and Markley, 1951).

2.3.3 Bellier tests

Bellier tests are used to detect the presence of sesame oil. It is based on the reaction of (a) ammonium vanadate and sulfuric acid gives a green color to greenish black, (b) formaldehyde and sulfuric acid gives a bluish black color and (c) resorcinol and nitric acid gives a bluish green color (Budowski and Markley, 1951).

2.3.4 Kreis "sesazo reaction"

Kreis "sesazo reaction" gives a red colour when sesame oil is shaken with an aqueous alkali solution of diazonaphthionic acid. This reaction is generally used to demonstrate the presence of sesamol in sesame oil (Budowski and Markley, 1951).

2.3.5 Ambuhl test

When rancid sesame oil or a rancid fat containing sesame oil is subjected to the Villavecchia test, an indigo-blue color is obtained. If crimson-red color is

obtained, this indicates the presence of fresh sesame oil (Budowski and Markley, 1951).

2.3.6 Guarnieri test

Guarnieri test (Budowski and Markley, 1951) is used to detect the presence of sesame oil. Positive results is revealed by a blue or green colour when 1 ml of an oil is treated with 2-3 drops of hydrogen peroxide prepared in diethyl ether followed by the addition of nitric acid (specific gravity 1.40).

2.3.7 Jacobson test

A positive Jacobson test result gives a greenish yellow colour on treating sesame oil with a mixture of perchloric acid and hydrogen peroxide. The same colour is obtained with pure sesamin and it can be used for the colorimetric determination of this substance (Budowski and Markley, 1951).

2.3.8 Tocher's test

Tocher's test involves shaking of oil with a solution of pyrogallol in concentrated hydrochloric acid. Presence of sesame oil gives purple colour. The Association of Official Agricultural Chemists authorize the use of either Boudouin's, Villivecchia's and Tocher's test for the detection of sesame oil in edible oils and fats (Budowski and Markley, 1951).

2.3.9 Behner's test

Behner's test is used to detect the presence of sesame oil in other oils**.** Test involves in shaking of oil with a mixture of equal parts of sulfuric and nitric acids, whereupon a green colour is obtained if sesame oil is present (Budowski and Markley, 1951).

2.3.10 Baudouin test

The addition of raw sesame oil to vegetable oil products to the extent of not less than 5% insured their detection by the Baudouin test at a 10% level in butterfat. The Baudouin test is considered to be specific for sesame oil. This test involves shaking the fat with a solution of sugar and adding an equal volume of concentrated hydrochloric acid. If sesame oil is present, a crimson colour develops after the mixture is allowed to stand for 10 minutes.

Sharma (1992) studied the physico-chemical properties of ghee prepared from milk adulterated with vanaspati and animal body fats. He prepared adulterated samples by two methods, in the first method vanaspati was added to milk and ghee was prepared from this milk and the second sample was prepared with direct addition of vanaspati to ghee. Baudouin test was performed and results revealed that the method of adulteration affected the Baudouin test used for detection of vanaspati. At 5 and 10% levels of adulteration with vanaspati, the crimson colour produced in acid layer was less intense in case of indirect adulteration.

Parmar (2005) evaluated the quality of market ghee and Hariyani (2010) studied the physico chemical qualities of market ghee. They reported negative Baudouin test in the market ghee samples, which showed that the market ghee samples were not adulterated with vanaspati.

Shukla *et al.* (2005) reviewed the detection of adulteration in edible oils using simple, rapid and reliable colour tests such as Sodium azide test, Modified nitric acid test, Azo dye test, Baudouin test, Hexabromide test, Halphen's test, Molybdate method and solvent partition test. These are performed for agremone oil, physically refined rice bran oil, sesame oil, linseed oil, cottonseed oil, castor oil and palmolein respectively with sensitivity level as 0.1%, 0.1%, 2.5%, 0.2%, 0.1%, 0.5% and 2% respectively.

2.3.11 Sesamin test

Sesame oil contains lignans like sesamin and sesamolin which can be used as tracer. In sesamin test, oil is mixed with furfural solution prepared in acetic anhydride and allowed to settle down. Further, few drops of sulphuric acid are added to the deposits. The development of greenish-blue colour indicates the presence of sesame oil (Codex, 1999). A dark blue coloration with a slight greenish tint develops immediately if sesame oil is present. The blue colour is progressively paler and the period required for the complete development of the colour is increased if lower amount of sesame oil is present.

Thirumala *et al.* (1968) developed a reaction which gave a characteristic colour with sesame oil. When trichloroacetic acid is added to sesame oil, an initial blue colour turns green after a few hours. One milliliter of 50% solution (v/v) of

sesame oil in petroleum ether is transferred to a test tube and 2 ml of chloroform solution of trichloroacetic acid (40% concentration, w/v) is added to it. A faint blue colour develops after a few seconds.

2.3.12 Modified Villavecchia test

Lee *et al.* (2013) examined the modified Villavecchia test for the detection of sesame oil. The test is based on the reactivity of sesamol and sesamolin with furfural under acidic conditions. Chromogenic products of the Villavecchia test with sesame oil prepared from different varieties of sesame seeds had different absorbance intensities at 520 nm. The absorbance intensities were positively correlated with the content of sesamolin in sesame oil. Roasting conditions affected the content and concentration of lignans in sesame oil and consequently the corresponding chromogenicity of the Villavecchia test. They reported that roasting of seeds at 230ºC for 5 min caused a significant loss of sesamolin in oil and the level of sesamol increased.

2.4 Study on oxidative stability of sesame oil

2.4.1 Effect of processing

High antioxidative activity of sesaminol was found during the industrial bleaching process of unroasted sesame seed oil. Sesaminol was a minor constituent isolated from acetone extract of sesame seed. It was shown that sesamolin in unprocessed sesame oil is the source of sesaminol and the formation of sesaminol was confirmed by the model experiment with corn oil to which sesamolin had been added. The antioxidative activity of sesaminol was roughly equal to those of sesamol and γ-tocopherol by the thiocyanate method. It appears that the antioxidative activity of refined unroasted sesame oil is mainly attributed by sesaminol (Fukuda *et al.* 1986).

Yoshida *et al.* (1995) studied variations in the composition of various acyl lipids, tocopherols and lignans in roasted sesame seeds oil. In this study seeds from various strains of cultivated *Sesamum indicum* Linn (colour of seeds: black, brown and white) were exposed to microwave roasting for 16 and 30 min at a frequency of 2450MHz and were studied not only for different acyl lipids and their fatty acid compositions, but also for the contribution of antioxidants to the oxidative stability of the oils. The sesame lignans amounted over 80% of the

original level. The results revealed that the oxidative stability of the oils would probably be due to the synergism between endogenous antioxidants and browning substances produced during microwave roasting.

The lignan contents of sesame oils were analysed and it was reported that mean of total lignans in sesame oil was 11.5 mg/g. Heating of sesame oil at 180ºC for 4 min did not change the total content of lignans but the level of sesamol increased after heating at 180ºC for 20 min. Heating at 200ºC for 20 min caused a significant loss of sesamolin and sesamol. Cooking at temperatures above 200ºC resulted in loss of some lignans but sesamin is relatively heat-stable (Wu, 2007).

Consumption of lignan rich food is presumed to have positive effects on human health. It is important to investigate the changes of the lignan content during processing. In this study, unheated and heated sesame seeds, sesame products, rye grains, rye flour, rye bread and flax seeds were extracted by sonication with ethanol:water (70:30, v/v) or sodium methoxide. The extracts were additionally hydrolysed enzymatically (b-glucuronidase/arylsulphatase, cellulase), the compounds separated on a reversed phase column by gradient elution and detected by UV/ESI-MS in the negative ionisation multiple reaction monitoring mode (MRM). Secoisolariciresinol, lariciresinol, pinoresinol, 7-hydroxymatairesinol, syringaresinol, isolariciresinol, secoisolariciresinol diglycoside, lariciresinol monoglycoside, pinoresinol mono-, di- and triglycoside, sesaminol, sesaminol triglycoside, sesamolinol and sesamolinol diglycoside were identified. Moderate heating at 100ºC did not degrade the lignan aglycones and glycosides in dry foods. In contrast, heating was responsible for the better extractability of the lignans. The degradation of the lignans in sesame seeds and rye was observed when the samples with high moisture content were heated at 100ºC. Higher roasting temperatures caused degradation of glycosides. Lignans degraded rapidly in sesame seeds and rye but not in flax seeds at 250ºC (Gerstenmeyer *et al.* 2013).

Changes in quality parameters were evaluated for refined sesame and soybean oil upon microwave heating. Soybean oil was more extensively oxidized by microwave heating than the other oil probably as a consequence of absence of natural antioxidants. Microwave heating also induced alterations in heating

profiles of the oils [Acid value of (0.67±0.04) and (1.8±0.01); peroxide value of (1.86±0.09) and (37.83±0.23); p-anisidine value of (16.46±0.12) and (18.23±0.23) after 10 minutes for sesame and soybean oils respectively]. Presence of frying components in soybean oil like polyunsaturated fatty acids enhanced thermo-oxidation and resulted in its poor stability (Ghosh *et al.* 2014).

The lignans in sesame seed oil could be categorized into two types: the inherent lignans (sesamin, sesamolin) and the lignans (e.g. sesamol, sesamolinol, etc.) that are mainly formed during oil production process. Sesame seed oil contains high contents of unsaturated fatty acids and even a small amount of free fatty acids, but it shows remarkable stability than other dietary vegetable oils. The better stability of sesame seed oil against autooxidation is not only because of the inherent lignans and tocopherols, but also by browning reaction products which produces during roasting. Also, there is an excellent synergistic effect among these three kinds of components (Wan *et al. 2015*).

2.4.2 Study on antioxidant activity of sesame lignans

Antioxidant activity of methanolic extract of sesame cake was evaluated in soybean, sunflower and safflower oils using the Schaal oven method and differential scanning calorimetry (DSC) analysis. Results showed that sesame cake extract (SCE), at concentrations of 5, 10, 50 and 100 ppm in vegetable oils could significantly ($P<0.05$) lower the peroxide value, diene value and p-anisidine value of oils during storage at 60ºC. The study also indicated a better antioxidant effect for sesame cake extract than Butylated hydroxy toluene (BHT) at 200 ppm (Suja *et al.* 2004).

Lignan compounds were extracted from roasted sesame oil and their effect on the autoxidation of methyl linoleate (ML) was studied. Sesamol, sesamin and sesamolin extracted from roasted sesame oil were added to ML which was oxidized at 60ºC for 18 hours in the dark. α-tocopherol was separately added to ML for a reference antioxidant. Degree of ML oxidation was monitored by conjugated dienoic acid (CDA) contents and *p*-anisidine value (PAV) by AOCS methods and ML retention was determined by gas chromatography. CDA contents and PAV of samples increased with the oxidation time at 60ºC in the dark and ML decreased. Sesamol, sesamin and sesamolin added samples

showed lower CDA contents, PAV, and ML loss than the samples without lignans during oxidation in the dark, which indicated that lignan compounds lowered the ML autoxidation. The antioxidant activity of sesamol was significantly higher (P <0.05) than that of sesamin, sesamolin and α-tocopherol. Sesamol, sesamin and sesamolin extracted from roasted sesame oil decreased ML autoxidation by lowering CDA formation and decomposition of primary oxidation products of ML. The antioxidant activity of sesamol was the highest and it was superior as compared to α-tocopherol. Sesamol, sesamin, and sesamolin degraded during the ML autoxidation with the highest degradation rate in sesamol. The degradation rate of sesamol was lower than that of α-tocopherol (Lee and Choe, 2006).

The effect of sesame lignans on the thermal and storage stability of edible vegetable oils (soybean oil, sunflower oil and rice bran oil) was studied by determining the total free radical scavenging activity using $DPPH^{\bullet}$, total tocol retention, lignan profile and poly unsaturated fatty acids (PUFA) composition. The order of radical scavenging activity and retention of total tocols of oils heated up to 120 min at frying temperature were rice bran oil = soybean oil > sunflower oil and rice bran oil > soybean oil > sunflower oil, respectively. Heating soybean oil and sunflower oil at frying temperature after addition of 1.2% lignans increased radical scavenging activity of soybean oil to a greater extent than that of sunflower oil but increased retention of total tocols observed only in soybean oil. Addition of lignans did not further increase the radical scavenging activity of rice bran oil. Heating oils with added lignans increased sesamol and decreased sesamolin while sesamin was relatively resistant to heat. These findings suggested that sesame lignans may have potential application as natural antioxidants in the edible oil and food industry (Hemalatha, 2007).

The oil extracted from sesame seed kernel was stored in white plastic containers using different antioxidants like Butylated hydroxy anisole (BHA), Butylated hydroxy toluene (BHT) and propyl gallate (PG) at various percentages (0.01-0.05%) at room temperature for 12 months. The results were evaluated after 2 months in terms of free fatty acid (FFA), iodine value and peroxide value. It was observed that the rate of increase in free fatty acid (FFA) was appreciably lower in all the three oils stored with 0.02% BHA, BHT and PG than other oils

containing percentage of 0.01, 0.03, 0.04 and 0.05 of antioxidants. The rate of increase in free fatty acid (FFA) of the oil containing 0.02% of BHA was found to be the lowest (0.1372). The iodine values of the oils were not influenced by any antioxidants of any percentages but the rate of increase in peroxide value of the oil containing 0.02% of BHA was also found to be significantly lower (Rahman *et al.* 2008).

2.5 Conclusion of the review of literature

Extensive study of literature showed that sesame lignans (sesamin and sesamolin) which are the tracer compounds found in sesame oil and are inherent compounds of sesame oil. These compounds are used as indicator to confirm the presence of sesame oil, but they tend to degrade with different processing as well as during the storage as reported in literature. Presence of sesamolin indicates the adulteration of vanaspati in ghee which can be detected by Baudouin test but as described in literature, sesamolin tends to degrade with different processing parameters which may lead to faulty results while detection of vanaspati. Whereas, sesamin is more stable than sesamolin during storage and in different processing conditions. There is a possibility to detect the presence of vanaspati in ghee during storage on the basis of sesamin (Sesamin test). Sesamin and Baudouin test are used to detect presence of vanaspati in ghee but, no work has been done to analyze the efficacy of Baudouin and sesamin test during storage. Due to the better stability of sesamin, detection of vanaspati based on sesamin would be a better alternative than sesamolin during storage. The research work was carried out to ascertain the efficacy of Baudouin and Sesamin test during storage.

CHAPTER –3

Materials & Methods

MATERIALS AND METHODS

The present investigation was planned to evaluate the efficacy of Baudouin and Sesamin test to detect vanaspati/ hydrogenated vegetable oil in ghee during storage. Stored adulterated ghee samples were subjected to Thin Layer Chromatography (TLC) as well as High Performance Liquid Chromatography (HPLC) to estimate the degradation of tracer compounds (sesamin and sesamolin) and correlating them with the results obtained for Baudouin and Sesamin test in adulterated ghee samples during storage. Materials and the methodologies used during the entire course of study are included in this chapter.

3.1 Materials

3.1.1 Chemicals and Reagents

All the reagents used were of “Analytical Reagents” grade unless otherwise specified.

Acetic anhydride, sodium thiosulphate, benzene, hydrochloric acid and sulphuric acid (Qualigens Fine Chemicals, Mumbai, India.), potassium dichromate, potassium hydroxide pellets and potassium iodide (Sisco Research Laboratories Pvt. Ltd, Mumbai, India), soluble starch (Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India), diethyl ether, methanol (HPLC grade),chloroform (Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India), ethanol absolute(S.D. Fine-chem Ltd., Mumbai, India), water (HPLC grade), furfural, sesamin and sesamol standards (Sigma Aldrich St. Louis, MO, USA), sesamolin standard (Cayman Chemicals, Michigan 48108 USA).

3.1.2 Glassware and Plastic ware

All volumetric flasks, pipettes and burettes were class “A”. Burette (50 ml), funnels (small and large), 50 ml graduated centrifuge tubes were purchased from Abdos Lab tech Private Ltd. New Delhi, India. Sample bottles (50 g) were procured from Axiva Sichem Biotech, New Delhi, India. Packaging material for ghee was procured from Swastik Industries, India. Measuring cylinders (10, 50,

100, 250, 500 and 1000 ml), pipettes (5 and 10 ml), porcelain dish, separating funnels (250 and 500 ml), volumetric flasks (5, 10, 50, 100, 250, 500 and 1000 ml, low actinic and/or clear), glass bottles with stopper (100 ml), glass test tubes with stopper and TLC chamber were purchased from Borosil India Ltd., Mumbai, India.0.22 µm cellulose acetate syringe filter was procured from Millipore India Pvt. Ltd., Bengaluru, India.

3.1.3 Specification of packaging material

Two different type of packaging material (multilayer pouches and sample bottles) were used for packaging of adulterated ghee samples for storage at 37ºC whereas, glass bottles with stopper were used for storage at 80ºC (accelerated storage study).

3.1.3.1 Multilayer pouch

Outer bag (double layer) polyester 12 micron+ low density polyethylene film (LDPEF) 300 gauge printed in multi-colour (invert printing), and inner bag was made from food grade low density polyethylene (LDPE) plain .

3.1.3.2 Sample bottles

Sample bottles were made of polystyrene with polypropylene lid were used for storage at 37ºC whereas, glass bottles with stopper were used for storage at 80ºC

3.1.4 Normal phase TLC plates

TLC Silica Gel 60 F_{254}aluminum 20×20 Sheets (Merck specialities private Ltd., India).

3.2 Equipments

3.2.1 Autopipettes : (10-100µl and 100-1000 µl): Tarsons Products Pvt. Ltd.,Kolkata, India.

3.2.2 Cream separator: Kamdhenu, KD-60E, Benny Impex, New Delhi, India

3.2.3 Vacuum filtration assembly (1000 ml): Schott Duran, Riviera, Wertheim, Germany

3.2.4 Ultrasonicator: SONICS, Vibra Cell, Model VCx750, USA

3.2.5 Boiling water-bath: Tempo Industrial Corporation, Bombay

3.2.6 BOD Incubator: Model NSW-152, Narang Scientific Works Pvt. Limited, Delhi, India

3.2.7 Refrigerated centrifuge: Kubota 6500, Kubota Corporation, Tokyo, Japan

3.2.8 Weighing balance: Model BP 221S, Sartorius India Pvt. Ltd. Mumbai, India

3.2.9 Water purifier: Sartorius arium pro, Sartorius India Pvt. Ltd. Mumbai India

3.2.10 HPLC system and accessories:

Waters High Pressure Liquid Chromatography 515. It comprised of pump control module II with two Waters 515 HPLC pumps; rheodyne manual injector, temperature controlled column compartment and Waters 2475 Fluorescence Detector, sample loop 20 µl, chromatograms were analyzed using Empower 2 software. (Milford, Massachusetts, USA)

3.2.11 Syringe driven filter unit: (33 mm, 0.22 µm pore size) Millex, Millipore, Billerica, Massachusetts, USA

3.2.12 Hamilton microlitre syringe (25 µl capacity) (for manual injections): Hamilton Company, Nevada, USA.

3.3 Methodology

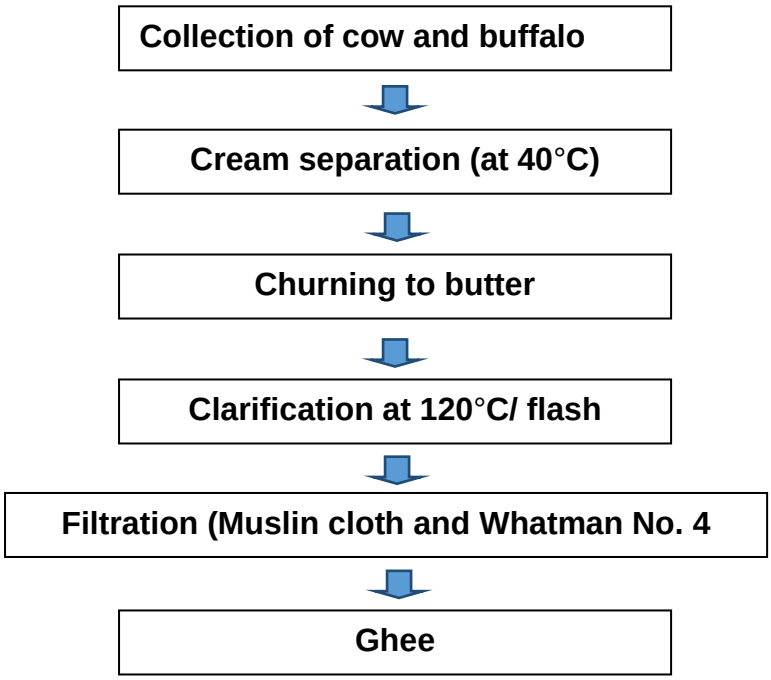
3.3.1 Collection and preparation of samples

3.3.1.1 Collection of milk and preparation of ghee

Cow and buffalo milk used for the preparation of respective ghee samples were collected from the experimental dairy of National Dairy Research Institute, Karnal. Cow milk was a mixture of the milk obtained from the herd of Karan Swiss, Karan Fries, Gir, Sahiwal and Tharparkar breeds. Buffalo milk used was from Murrah breed only. Samples of cow/buffalo ghee were prepared by creamery butter method (De, 2015). Soon after the collection of milk samples, these were warmed to 40°C and cream was separated using mechanical cream separator. The cream was pasteurized at 77°C for 5 minutes and cooled to room temperature. Butter was prepared under standard conditions by churning the cream using hand operated butter churn. The butter was then heated on direct

flame in a stainless steel vessel and clarified into ghee with continuous stirring at temperature of 120°C/flash. Ghee was then filtered through 6-8 fold muslin cloths followed by whatman No.4 filter paper.

The flow chart for the preparation of mixed ghee (cow and buffalo) was as follows:



For preparation of mixed ghee, cow and buffalo milk was mixed in 1:1 ratio.

3.3.1.2 Procurement of vanaspati

Vanaspati (Dalda) was used as the adulterant fat in the present study. It was procured from local market of Karnal.

3.3.1.3 Confirmation of sesame oil in vanaspati

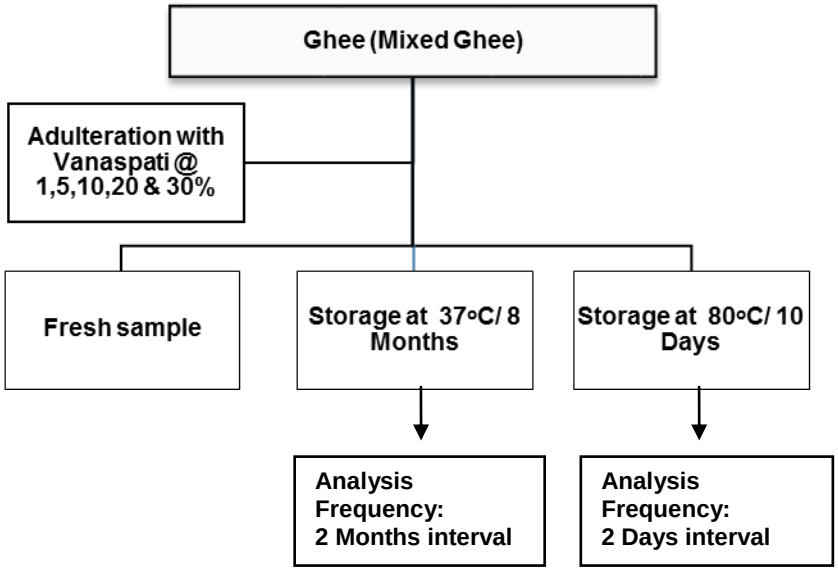
According to Vegetable Oil Products Control Order (1947), addition of 5 % sesame oil is compulsory in vanaspati. Presence of lignans like sesamolin and sesamin in sesame oil can be used as tracer compounds, which could be further detected by Baudouin and Sesamin test, respectively. A positive Baudouin test shows development of red coloured complex which indicates presence of sesamolin. A positive Sesamin test shows development of bluish

green colour which indicates the presence of sesamin. To confirm the presence of sesame oil, procured vanaspati samples were subjected to Baudouin and Sesamin test. Vanaspati with positive Baudouin and Sesamin test was selected as adulterant fat to prepare adulterated ghee samples for the present study.

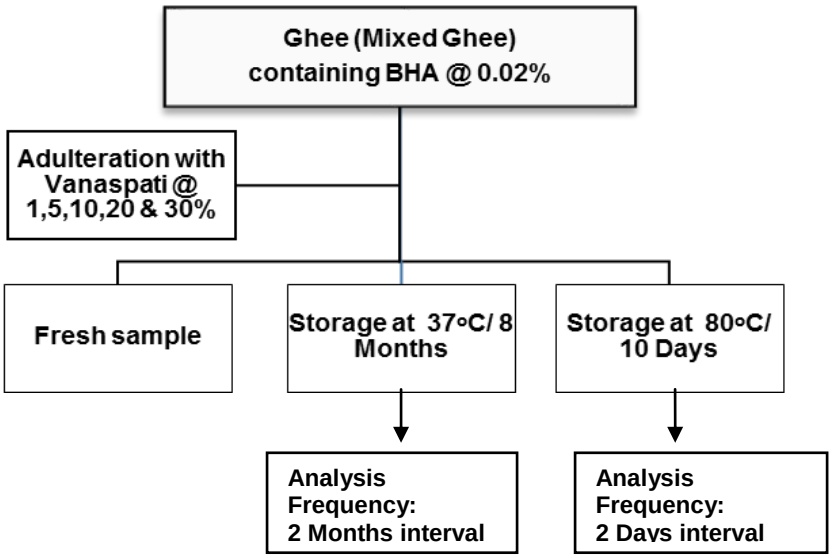
3.3.1.4 Preparation of ghee samples adulterated with vanaspati

Ghee prepared with and without BHA was used for the study. For the preparation of adulterated ghee samples, ghee and vanaspati were heated separately to 60-70°C for 10 min before mixing. The adulterant fat was added to mixed ghee individually at the rate of 1, 5, 10, 20 and 30% levels. Two different time-temperature combinations (37°C for 8 months and 80°C for 10 days) were used in the present study. Adulterated ghee samples were subjected to accelerated storage conditions (80°C for 10 days) to determine the long term effects of storage on Baudouin and Sesamin test within a shorter time which is shown in flowchart.

3.3.1.4.1 Ghee samples (without BHA) adulterated with vanaspati



3.3.1.4.2 Ghee samples (with BHA) adulterated with vanaspati



3.4. Peroxide value

Peroxide value of ghee samples was determined by the method as described in IS: 3508 (1966).

3.4.1 Preparation and standardization of 0.1 N $Na_2S_2O_3$ solution (stock solution)

Approximately 25 g of sodium thiosulphate ($Na_2S_2O_3$) crystals was dissolved in well boiled and CO_2 free water and volume was made up to 1000 ml. The solution was stored in a dark coloured reagent bottle and standardized as follows:

About 5 g of finely ground potassium dichromate ($K_2Cr_2O_7$) was accurately weighed and transferred to a clean volumetric flask (1000 ml). Some distilled water was poured into the volumetric flask and it was thoroughly shaken and finally volume was made up to the mark. The solution was kept in a cool, dry place. 25 ml of this solution was pipetted out into a clean glass stoppered 250 ml conical flask. 5 ml of concentrated HCl and 15 ml of 10% potassium iodide solution were added. The solution was then allowed to stand in the dark for 5 minutes and the mixture was titrated against $Na_2S_2O_3$ solution (0.1 N as

prepared above) using 2 ml of starch solution as an indicator towards the end. The end point was the change in colour from blue to green.

The normality of $Na_2S_2O_3$ solution was calculated as follows:

$$\text{Normality of } Na_2S_2O_3 \text{ solution} = \frac{25 \times W}{49.03 \times V}$$

Where,

W = Weight of $K_2Cr_2O_7$ in g

V = Volume of $Na_2S_2O_3$ required for titration in ml

3.4.2 Preparation of 0.002 N solution of $Na_2S_2O_3$

Prepared freshly by diluting the previously prepared 0.1 N $Na_2S_2O_3$ stock solution to obtain 0.002N solution of Na_2S_2O.

3.4.3 Saturated potassium iodide (KI) solution

Ten milliliter of water was boiled for 5 minutes and allowed to cool. To a portion, potassium iodide was added to ensure a saturated solution (~10 g KI in 6 ml water).

3.4.4 Starch indicator (1%)

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 tight stoppered bottle.

3.4.5 Mixed solvent

Glacial acetic acid: Chloroform, 2:1 (v/v).

3.4.6 Procedure

One gram of ghee was weighed into a 250 ml glass stoppered Erlenmeyer flask. 20 ml of mixed solvent was added and flask was then swirled until the dissolution of fat. About 0.5 ml of saturated KI was then added to the flask and kept undisturbed for 1 minute. The mixture was heated on a boiling water bath up to boiling and tip of the flask was closed with finger after vapours started to form and boiling was continued for 30 seconds. Generated vapours

were condensed under a stream of running tap water. 25 ml of distilled water was added followed by 0.5 ml of 1% starch indicator. At this point dark blue/brown colour appeared. The mixture was then titrated against 0.002 N $Na_2S_2O_3$ until disappearance of colour. Blank without any sample was also run simultaneously.

3.4.7 Calculation

The peroxide value as milliequivalents of peroxide oxygen per kg of fat was calculated as:

$$\text{Peroxide value} = \frac{(S-B) \times N \times 1000 \times 8}{W}$$

Where,

S= Volume of $Na_2S_2O_3$ required for titration of sample in ml

B= Volume of $Na_2S_2O_3$ required for titration of blank in ml

N= Exact normality of $Na_2S_2O_3$ solution

W= Weight of sample

Note: The peroxide value as millimoles of oxygen per kg of fat was calculated as peroxide value/16.

3.5 Baudouin Test

Baudouin test for ghee was performed as described in IS 548-2 (1976).

3.5.1 Reagents

Concentrated hydrochloric acid (Relative density=1.19)

Preparation of furfural solution (2% v/v): Two milliliter of furfural was transferred to a 100 ml volumetric flask and volume was made up with 95% ethanol.

3.5.2 Procedure

Five milliliter of the melted ghee was transferred to a 25 ml test tube provided with a glass stopper, 5 ml of hydrochloric acid and 0·4 ml of furfural solution were added to the ghee sample. The glass stopper was fitted in the test

tube and the contents were shaken vigorously for 2 min. The mixture was left undisturbed for separation of two layers. The development of a pink or red colour in the acid layer indicated the presence of sesame oil. It was confirmed by adding 5 ml of water and shaking again, the persistence of colour in acid layer indicated the presence of sesame oil. Disappearance of colour indicated the absence of sesame oil.

3.5.3 Expression of Results: The result was expressed as positive or negative.

3.6 Sesamin test

Sesamin test for ghee was performed as approved by Codex (1999) with slight modification (i.e. concentration of furfural solution was changed from 0.35/ml to 0.00035/ml acetic anhydride).

3.6.1 Reagents

Concentrated sulphuric acid (relative density 1.84)

Preparation of furfural solution (0.035% v/v): 35 µl of freshly distilled furfural was transferred to a 100 ml volumetric flask and volume was made up with acetic anhydride.

3.6.2 Procedure

Ten milliliter of the melted ghee was transferred to a test tube and 5 ml of furfural solution was added to it. Stopper was fitted and the contents were shaken vigorously for approximately one minute. 1 or 2 ml of the deposit was transferred to a porcelain dish and 7 drops of sulphuric acid were added. The dish was gently shaken and left undisturbed for 5 min. The development of greenish-blue colour indicated the presence of sesame oil.

3.6.2 Expression of Results: The result was expressed as positive or negative.

3.7 Profiling of sesamin and sesamolin using chromatographic procedures

3.7.1 Preparation of methanolic potassium hydroxide solution (5%): About 50 g of potassium hydroxide pellets were dissolved in distilled water and volume was made up to 1000 ml in volumetric flask.

3.7.2 Preparation of aqueous potassium hydroxide solution (0.5N): About 28.05 g of potassium hydroxide pellets was dissolved in distilled water and volume was made to 1000 ml in a volumetric flask.

3.7.3 Phenolphthalein indicator: About1 g of phenolphthalein was dissolved in 5 ml of distilled water and then volume was made to 100 ml with absolute ethyl alcohol.

3.7.4 Preparation of sesamin, sesamol, and sesamolin Standards: sesamin, sesamol, and sesamolin stock standards were prepared at 1.0 mg/mL in methanol. The standards were thoroughly mixed and closely observed to ensure that the components were completely soluble in the methanol. These stock solutions were diluted with methanol to prepare standards of desired concentration and filtered through 0.45 micron syringe filter. All standards were stored at 4 to 7ºC.

3.8 Normal phase Thin Layer Chromatography of unsaponifiable matter

Normal phase Thin Layer Chromatography (TLC) was used to analyze the presence of sesame lignans in adulterated ghee samples. The procedure used for TLC is discussed below:

3.8.1 Extraction of unsaponifiable matter (USM) from ghee samples

Unsaponifiable matter from ghee samples was isolated as per the method of IS: 3508 (1966). Five gram molten fat sample was weighed in a 250 ml flat bottom flask followed by the addition of 50 ml of 5% methanolic potassium hydroxide (KOH) solution and 2-3 glass beads. The contents were thoroughly mixed. The flask was attached to reflux condenser properly and heated on a boiling water bath for 1hr. After completion of saponification the contents were transferred to 500 ml capacity separating funnel containing 150 ml water (Separating funnel was previously rinsed with water and diethyl ether).100 ml of diethyl ether was added to the separating funnel. Stopper was inserted into the funnel and shaken with intermittent removal of vapours for releasing the pressure. The funnel was allowed to stand until two layers separated. Lower aqueous methanolic soap layer was collected in beaker for further extraction and

upper etheral layer was transferred to another 250 ml separating funnel containing 20 ml of water.

Aqueous methanolic soap layer was extracted two times with 50 ml of diethyl ether. Collected etheral layer was transferred to a separating funnel and washed twice with 20 ml distilled water. Successive washing was performed (two times) with 20 ml of 0.5 N aqueous potassium hydroxide and 20 ml water. The washings were continued with water till the wash water was free from any soap. Phenolphthalein indicator was used to ensure the presence or absence of soap in the wash water. In case the soap is present in wash water, pink colour appears on adding the phenolphthalein indicator. The washed etheral layer was transferred to a conical flask by passing through anhydrous sodium sulfate for removing traces of moisture entrapped in the solvent during washing. The etheral layer was evaporated on the water bath maintained at 60ºC and finally transferred the flask to an oven maintained at 80ºC. The dried unsaponifiable matter obtained was then dissolved by adding 500 µl of chloroform and 500 µl of methanol and transferred to an eppendorf tube.

The method for the extraction of unsaponifiable matter is summarized below.

5 g molten fat sample

↓

50 ml of 5% methanolic KOH

↓

Refluxing on a boiling water bath for 1hr

↓

Contents were transferred to separating funnel containing 150 ml water

↓

Addition of diethyl ether (100 ml)

↓

Mixed properly with releasing the pressure

↓

Allowed to stand until two layers separated

↓

Lower aqueous methanolic soap layer subjected for further extraction

↓

Upper etheral layer transferred to another separating funnel containing 20 water

↓

Aqueous methanolic soap layer twice extracted with 50 ml of diethyl ether

↓

All collected etheral layers transferred to a separating funnel for washing

↓

Washed twice with 20 ml distilled water

↓

Washing (two times) with 20 ml of 0.5 N aqueous KOH and 20 ml water

↓

Washing with water till the wash water was free from any soap

↓

Evaporation of diethyl ether

↓

Unsaponifiable matter

3.8.2 Conditions for normal phase Thin Layer Chromatography (TLC) of unsaponifiable matter using silica Gel 60 F_{254} Plates as a Stationary Phase

Developing solvent consisting of chloroform: benzene: methanol (60:40:1 v/v/v) was added to a TLC glass chamber lined with filter paper. Chamber was saturated for about 15 min. 20 µl of the unsaponifiable matter solution (dissolved unsaponifiable matter in chloroform and methanol) was spotted on TLC silica gel plate at a distance of about 1 cm from the bottom along with solutions of standard sesamin, sesamol, sesamolin and mixture (sesamin + sesamol + sesamolin) as different spots and allowed them to dry in air. TLC plate was then placed in the developing chamber saturated with developing solvent till the solvent front had travelled about three-quarters of the length of the plate. The plate was then removed, dried, and sprayed with detecting reagent (20%sulphuric acid solution made in methanol) and kept at 110°C / 5-10 min and position of bands was compared with standards.

3.9 High Performance Liquid Chromatography (HPLC) of unsaponifiable matter

In order to analyze for the presence of sesame lignans in adulterated ghee samples, High Performance Liquid Chromatography (HPLC) technique was applied. The method used in the study discussed below:

3.9.1 Extraction of unsaponifiable matter (USM) from the fat samples

USM was extracted from ghee samples as described in section 3.8.1. The dried unsaponifiable matter obtained was dissolved in 500 µl of chloroform and 500 µl of methanol and filtered through 0.22µ syringe filter and subjected to HPLC analysis. Along with this, the reference standards of sesamin and

sesamolin of 0.2mg/ml concentration were also run on HPLC and peak detection was made with Fluorescence Detector (Ex.290, Em.320).

3.9.2 Analytical conditions for HPLC

The following analytical conditions were standardized for the detection of sesame oil lignans

System : Waters High Pressure Liquid Chromatography 515 (Milford, Massachusetts, USA) consisted of pump control module II with two Waters 515 HPLC pumps and chromatograms were analyzed using Empower 2 software

Column	:	C18 100 °A, 250×4.6 mm, Waters, USA
Column holder	:	Delta-Pack, Waters, Massachusetts, USA
Detector	:	2475 Fluorescence Detector (Ex.290, Em.320).
Phase	:	Reversed phase
Mobile phase	:	Methanol: Water (70:30)
Flow rate	:	0.8 ml/min
Run time	:	50 min
Injection volume	:	20 µl
Syringe filter	:	0.22 µm PVDC Millipore (33 mm)
Syringe	:	25 µl Blunt type (Hamilton)

3.9.3 Plotting of calibration curve of sesamin and sesamolin

6-point calibration curve were plotted for sesamin and sesamolin. Curves were prepared by injecting 0.001, 0.01, 0.05, 0.1, 0.15 and 0.2 mg/ml of standard solution of sesamin and sesamolin. Calibration curve were then drawn by plotting concentration against peak area and correlation coefficients (R^2) determined. Linear regression equation was determined for sesamin and sesamolin standards to establish the linearity of the system.

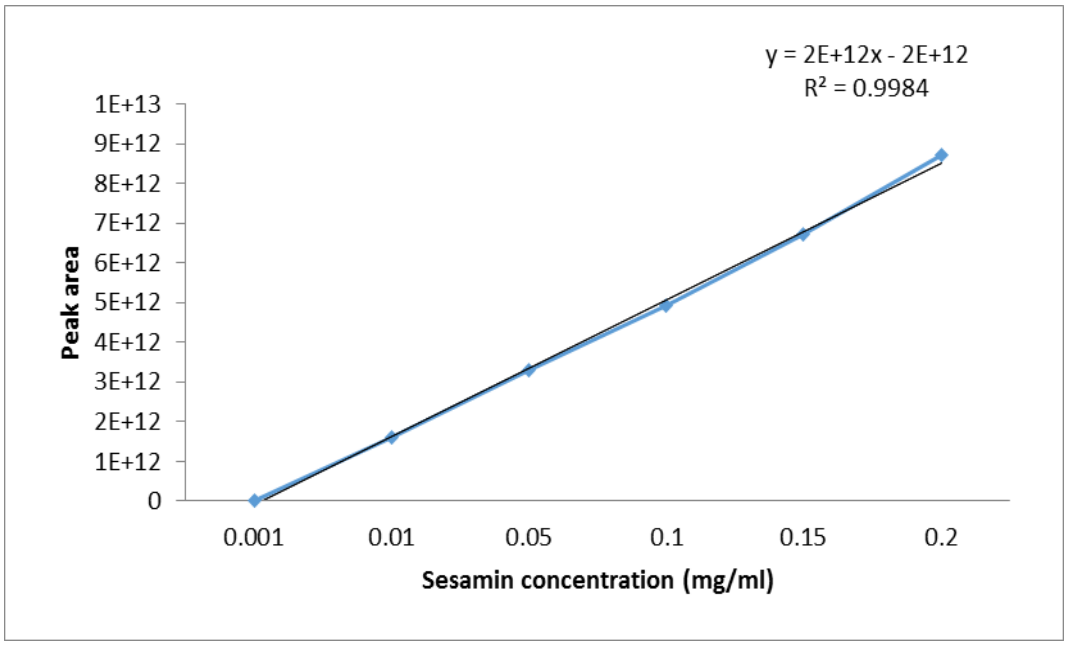


Fig 3.1 Standard curve for Sesamin

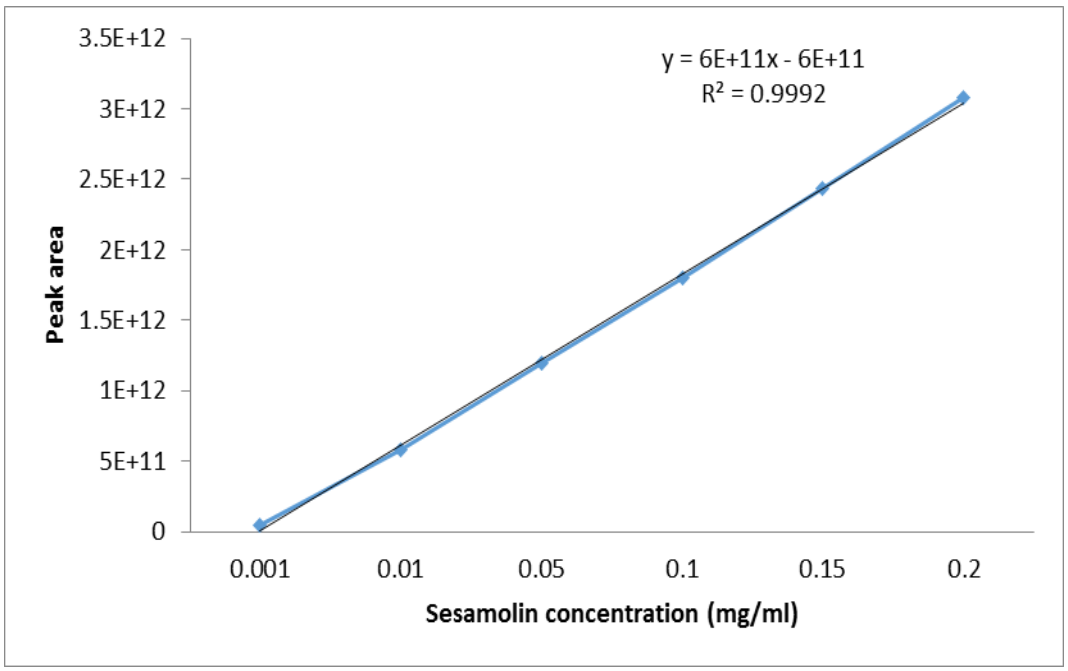


Fig 3.2 Standard curve for Sesamolin

CHAPTER –4

Results and Discussion

RESULTS AND DISCUSSION

Work was planned to evaluate the efficacy of Baudouin and Sesamin test to detect vanaspati/hydrogenated vegetable oil in ghee during storage. Ghee samples with and without addition of BHA (0.02%) were used for the present study to evaluate the effect of antioxidant on the degradation of tracer compounds (sesamin and sesamolin). Vanaspati was added to mixed ghee individually at the rate of 1, 5, 10, 20 and 30% levels, respectively for the preparation of adulterated ghee samples. The adulterated ghee samples were stored at 37ºC for 8 months and also at 80ºC for 10 days. The stored adulterated ghee samples were subjected to Baudouin test, Sesamin test, peroxide value and chromatographic analysis e.g. TLC and HPLC for the estimation of degradation in tracer compounds (Sesamin and Sesamolin) to evaluate the effect on Baudouin and Sesamin test during storage.

Adulterated stored ghee samples were analysed for following parameters:

- Sesamin test
- Baudouin test
- Peroxide value
- Profiling of sesamin and sesamolin by chromatographic procedures

4.1 Sesamin test

A colour based Sesamin test for the detection of sesame oil in vegetable oil has been approved by Codex (1999), in which development of bluish green colour indicates the presence of sesamin. This test has not been performed in case of ghee. However, on performing this test with ghee according to the procedure approved by Codex, the black colour obtained was unable to confirm the presence of vanaspati (containing sesame oil) in adulterated ghee. Therefore, to confirm the presence of vanaspati in adulterated ghee samples the test was modified as follows:

4.1.1 Optimization of analytical conditions for sesamin test in ghee

To optimize the sesamin test in ghee, concentration of furfural solution was optimised. Three different concentrations (0.35%, 0.1% and 0.035% furfural in acetic anhydride) of furfural solution were analysed for the proper colour development. The ghee samples adulterated with maximum concentration of vanaspati (30%) was used for the optimization of analytical conditions for sesamin test in ghee. It is evident from the Fig. 4.1 that 0.035% furfural solution gave appropriate colour development for the detection of vanaspati (containing sesame oil) in ghee. This optimized concentration of furfural solution was used in further analysis.

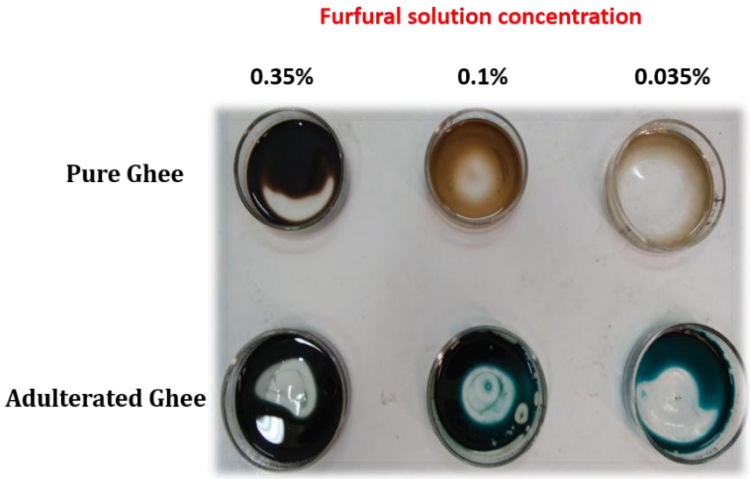


Fig 4.1 Effect of furfural concentration on Sesamin test

Results from the Fig 4.2 revealed that, Sesamin test was able to detect the adulteration of ghee adulterated with vanaspati at higher concentrations (10 to 30% vanaspati adulterated ghee), whereas at lower concentrations (i.e.1% and 5%) this test did not give promising results. This could be due to low amount of sesamin compound present in adulterated ghee samples containing lower amount of vanaspati.

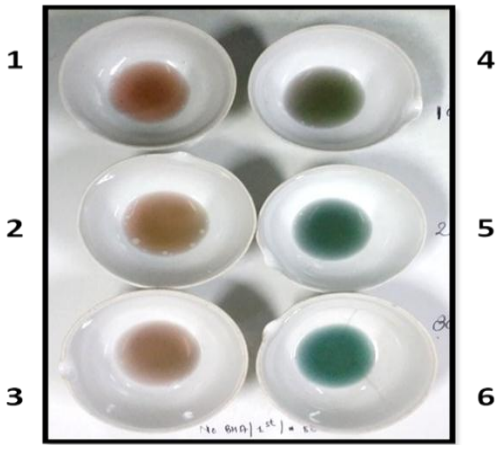


Fig 4.2 Sesamin test for adulterated ghee samples

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

4.1.2 Effect of storage on Sesamin test

The adulterated ghee samples were kept in sample bottles and multilayer pouches and then stored at 37ºC for 8 months. The reason behind selecting different type of packaging material was to examine the effect of packaging materials on degradation of sesamin and sesamolin. Accelerated storage of ghee samples was also carried out at 80ºC for 10 days to evaluate the changes occurring in sesamin content and its effect on Sesamin test. Samples which were subjected to accelerated storage conditions were kept in glass bottles.

4.1.2.1 Changes in Sesamin test during storage at 37ºC for 8 months

4.1.2.1.1 Sesamin test of fresh and stored adulterated ghee samples

Adulterated ghee samples were stored at 37ºC and tests were performed at interval of 2 months during the storage period of 8 months. Results of Sesamin test are depicted in Fig 4.7. It was evident from the Fig 4.3 to Fig 4.7 that adulterated ghee samples with and without BHA gave positive Sesamin test during storage for 8 months. No adverse effect or interference of BHA was observed on the results of Sesamin test for freshly prepared adulterated ghee samples. On the initial day of storage, ghee samples adulterated with vanaspati at higher concentrations (i.e. 10, 20 and 30% of adulteration) showed positive results for Sesamin test, whereas negative results for Sesamin test were

observed at lower concentrations of ghee samples adulterated with vanaspati (1 and 5%). It is also evident from the Fig 4.3 to 4.7 that, there was no adverse effect of storage period on adulterated ghee samples for Sesamin test. This could be due to higher stability of sesamin during storage, as the results were similar to the initial results. Yoshida *et al.* (1995) reported recovery of sesame lignans more than 80% of the original level after microwave heating. Hemalatha (2007) also reported that sesamin is more stable than sesamol and sesamolin. Moreover, there was negligible effect of packaging material and addition of BHA on Sesamin test during storage. Sesamin test gave positive results for 10, 20 and 30% adulterated ghee samples packed in sample bottles and multilayer pouches, as well as similar findings were obtained for the samples with and without BHA which might be due to negligible changes in the amount of sesamin during storage.

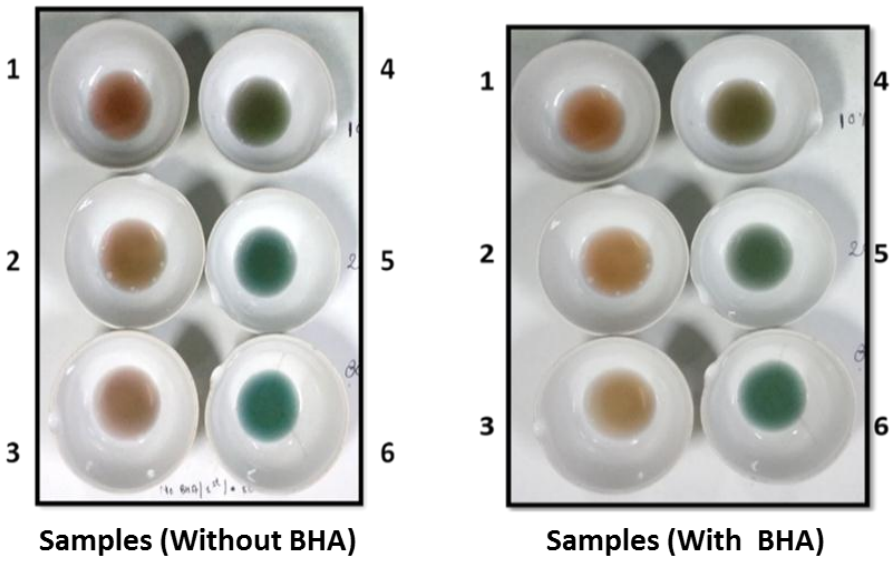
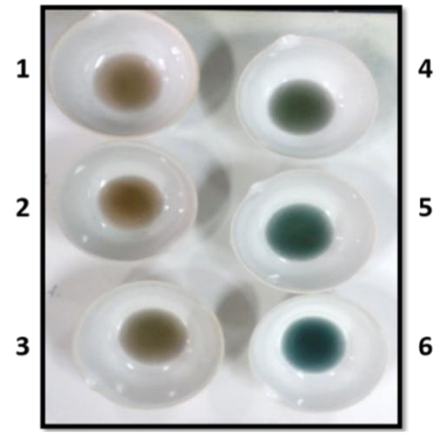
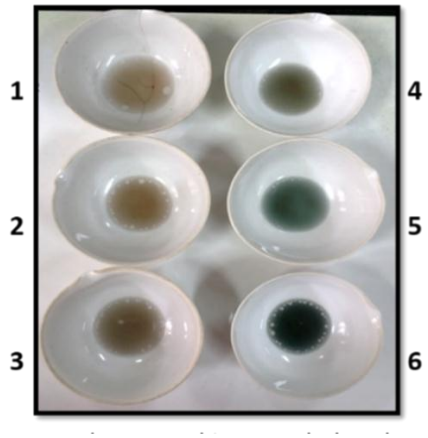


Fig.4.3 Sesamin test for adulterated ghee samples on 0 month of storage

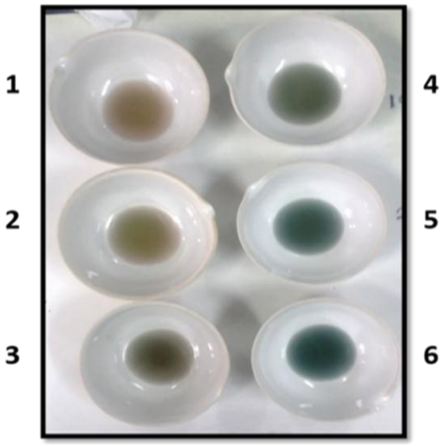
1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated and 6) 30% adulterated



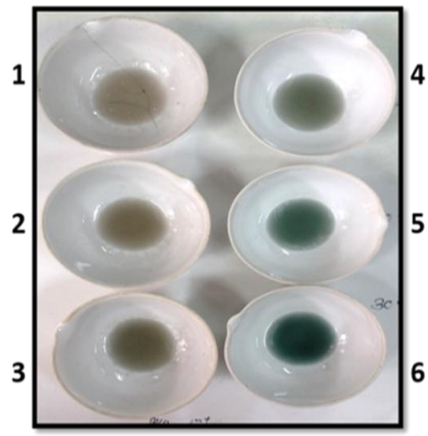
Samples stored in sample bottle
(Without BHA)



Samples stored in sample bottle
(With BHA)



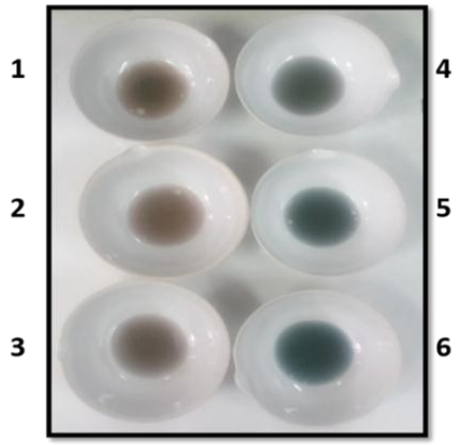
Samples stored in multilayer pouch
(Without BHA)



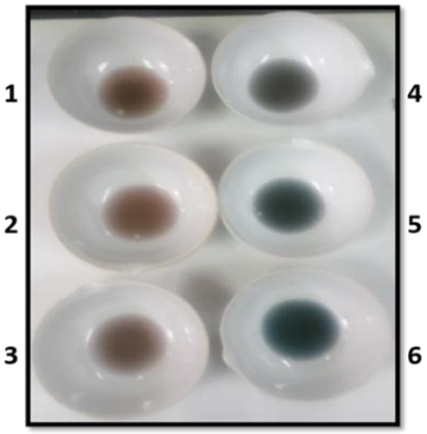
Samples stored in multilayer pouch
(With BHA)

Fig.4.4 Sesamin test for adulterated ghee samples on 2nd month of storage

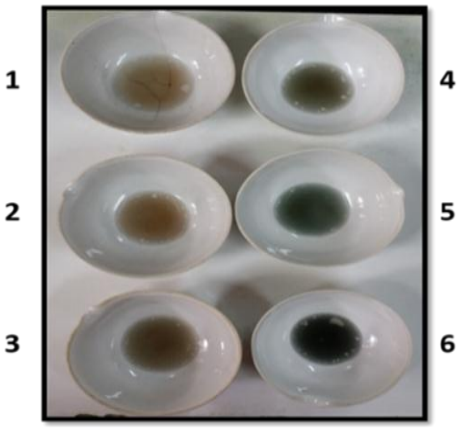
1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated



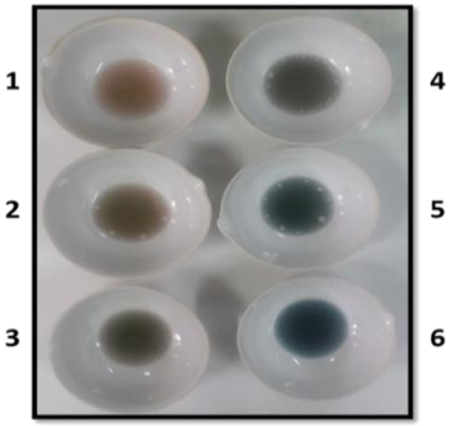
Samples stored in sample bottle
(Without BHA)



Samples stored in sample bottle
(With BHA)



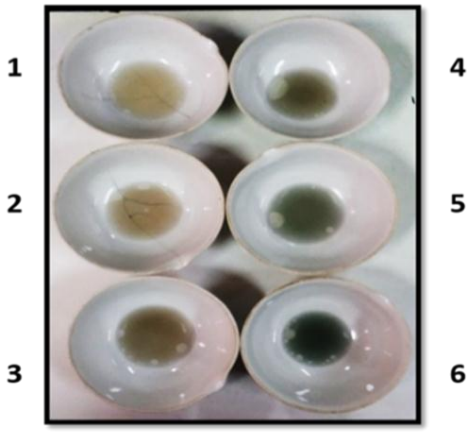
Samples stored in multilayer pouch
(Without BHA)



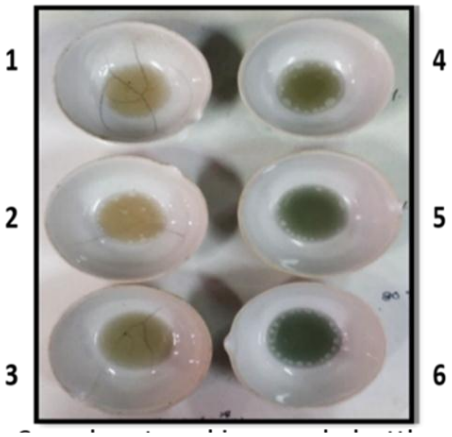
Samples stored in multilayer pouch
(With BHA)

Fig.4.5 Sesamin test for adulterated ghee samples on 4th month of storage

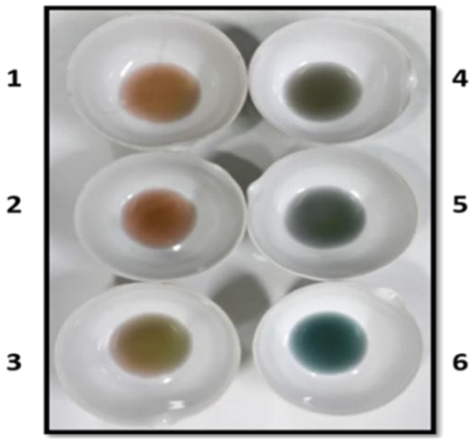
1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated



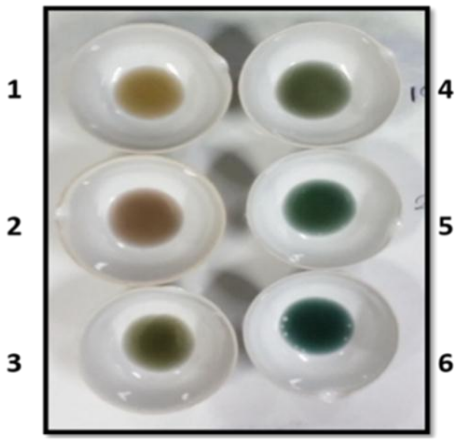
Samples stored in sample bottle
(Without BHA)



Samples stored in sample bottle
(With BHA)



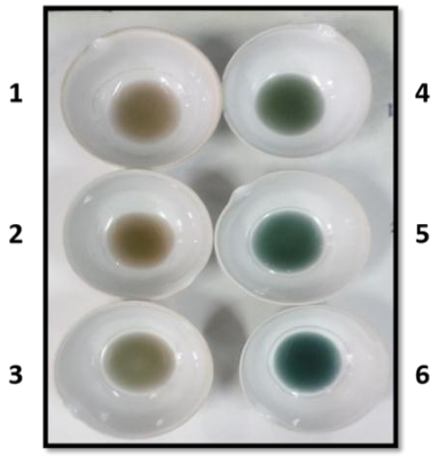
Samples stored in multilayer pouch
(Without BHA)



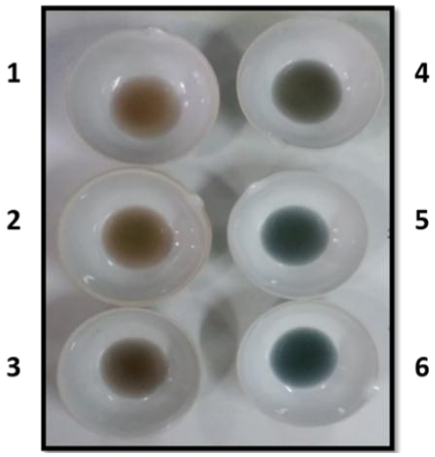
Samples stored in multilayer pouch
(With BHA)

Fig.4.6 Sesamin test for adulterated ghee samples on 6th month of storage

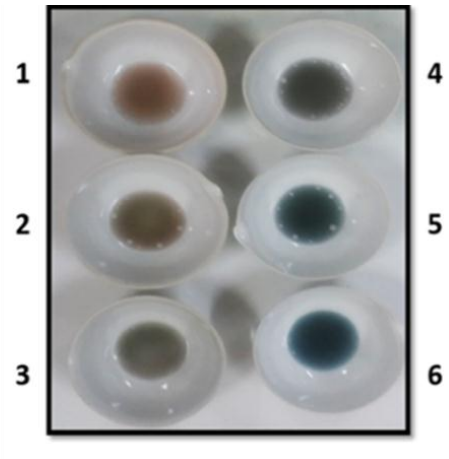
1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated and 6) 30% adulterated



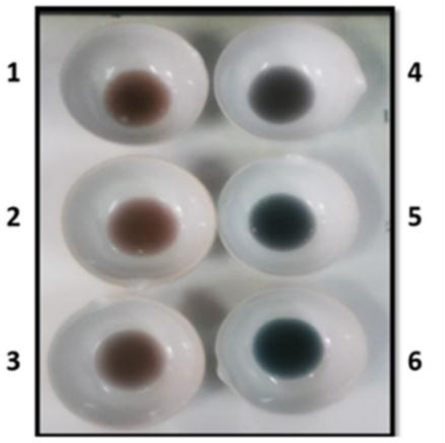
Samples stored in sample bottle
(Without BHA)



Samples stored in sample bottle
(With BHA)



Samples stored in multilayer pouch
(Without BHA)



Samples stored in multilayer pouch
(With BHA)

Fig.4.7 Sesamin test for adulterated ghee samples on 8th month of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

4.1.2.2 Changes in Sesamin test during accelerated storage at 80ºC for 10 days

To examine the changes in the amount of sesamin and its effect on Sesamin test in vanaspati adulterated ghee samples, it was stored at 80ºC for 10 days (accelerated storage conditions). Adulterated ghee samples (with and without addition of BHA) were stored at 80ºC for 10 days in glass bottles. Samples were subjected to Sesamin test at an interval of 2 days. It is evident from Fig 4.8 that freshly prepared samples showed positive results at 10% level of adulteration and also at higher concentrations (20 and 30%), whereas negative results were obtained at lower concentrations (1 and 5%). Positive results (Fig 4.8 to 4.12) of Sesamin test were obtained for 10, 20 and 30% adulterated samples after storage of 8 days at 80ºC. However, on 10^{th} day of storage, it was observed from Fig 4.13 that the test result was doubtful in case of 10% adulterated ghee samples (without BHA), whereas 20 and 30% adulterated ghee samples (without BHA) showed no adverse effect on storage. It could be inferred from Fig 4.13 that samples (with BHA) showed stable colour as compared to samples without BHA. The colour was stable in case of samples with BHA due to presence of BHA which might have prevented the degradation of sesamin. Rahman *et al.* (2008) observed significantly lower peroxide value in sesame oil containing 0.02% BHA which prevented auto-oxidation of sesame oil. It can therefore be concluded that BHA also prevented the auto-oxidation of the compound (i.e. sesamin and sesamolin) present in sesame oil. It can therefore be concluded that Sesamin test is very much applicable for the detection of vanaspati in ghee.

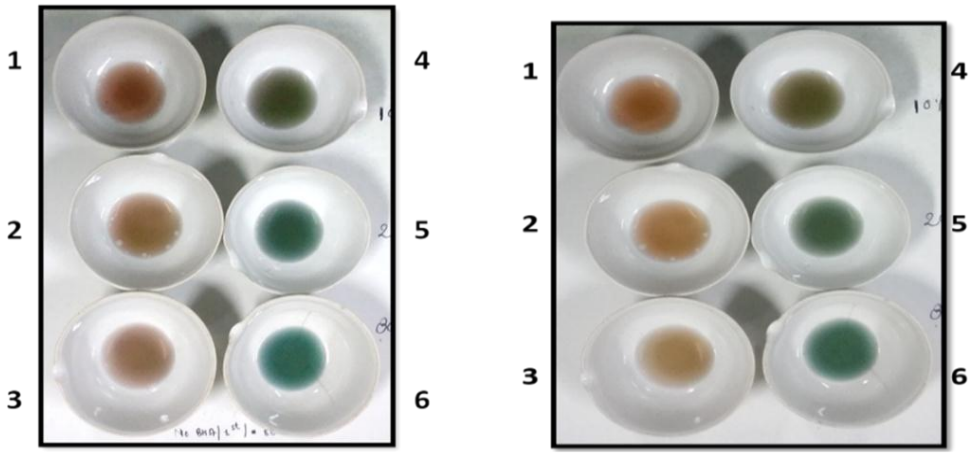


Fig.4.8 Sesamin test for adulterated ghee samples on 0 day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

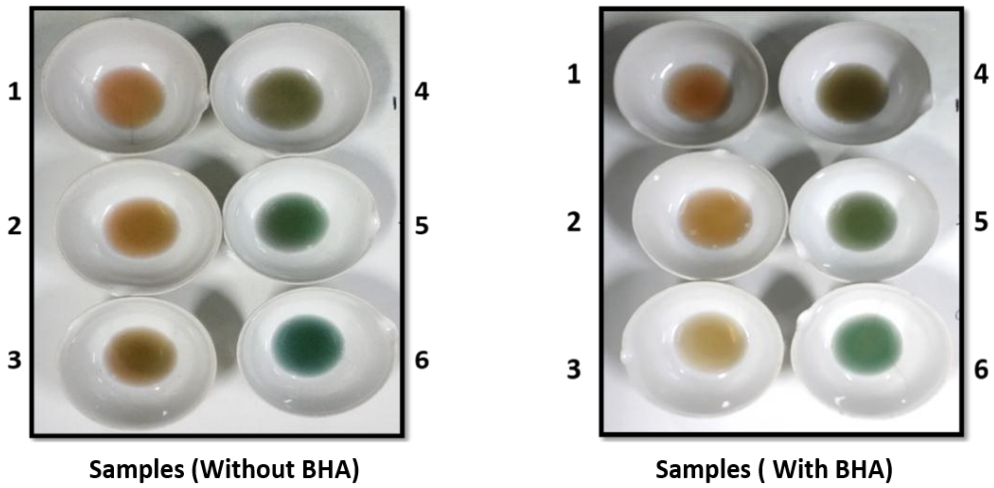


Fig.4.9 Sesamin test for adulterated ghee samples on 2nd day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

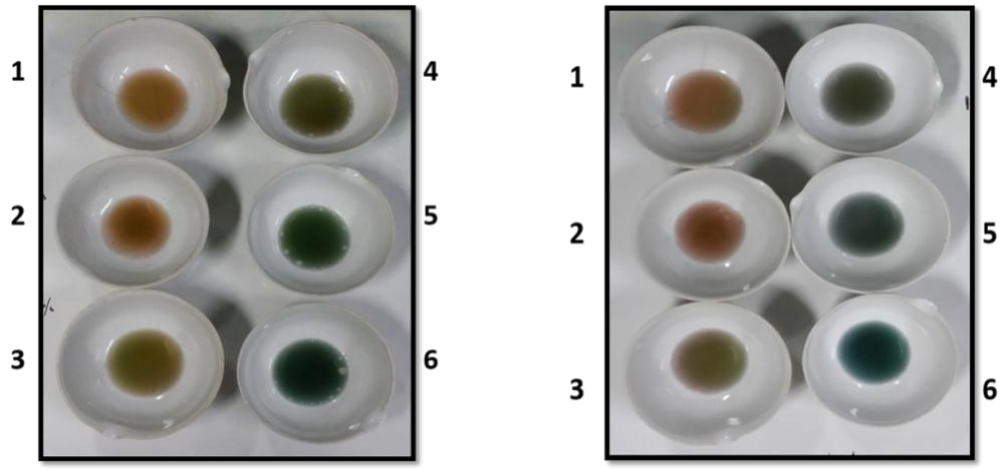


Fig.4.10 Sesamin test for adulterated ghee samples on 4th day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

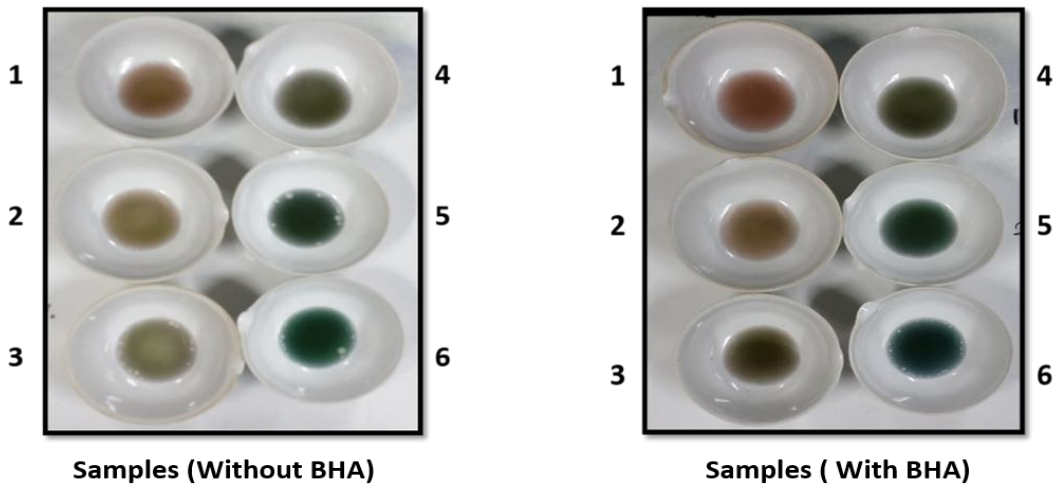


Fig.4.11 Sesamin test for adulterated ghee samples on 6th day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

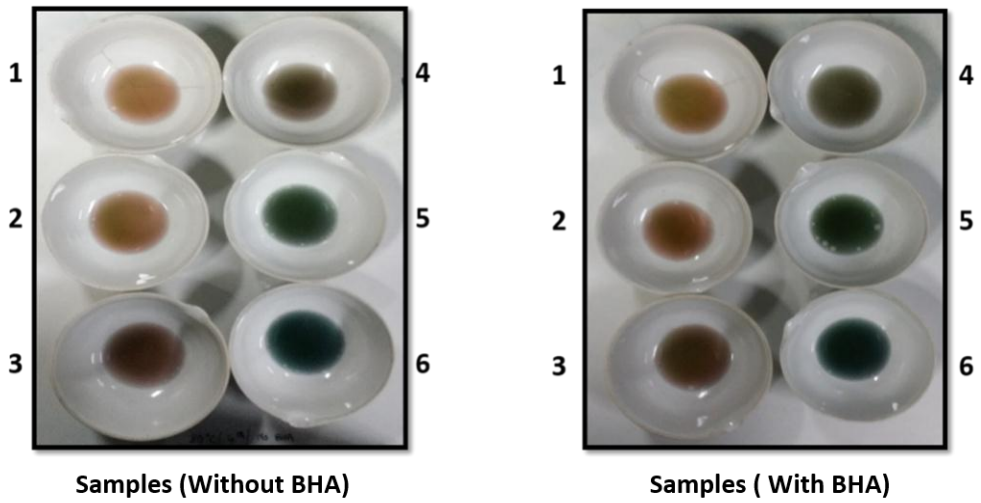


Fig.4.12 Sesamin test for adulterated ghee samples on 8th day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

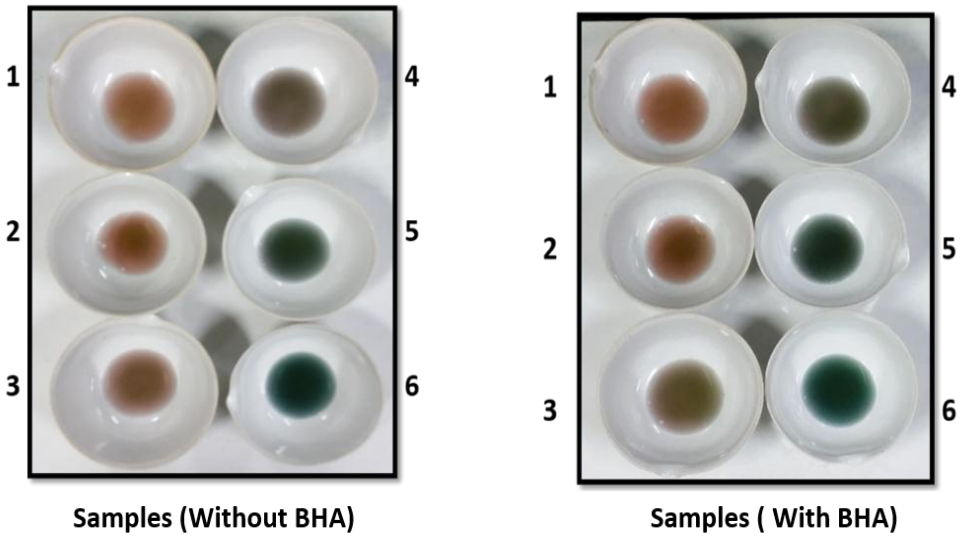


Fig.4.13 Sesamin test for adulterated ghee samples on 10th day of storage

1) Pure ghee, 2) 1% adulterated, 3) 5% adulterated, 4) 10% adulterated, 5) 20% adulterated, 6) 30% adulterated

4.2 Effect of storage on Baudouin test

A colour based Baudouin test for the detection of vanaspati in ghee has been described in IS 548-2 (1976). The adulterated ghee samples were subjected to Baudouin test before and after storage at 37ºC for period of 8 months. The effect of storage, packaging materials and addition of BHA were analysed during the storage period. Samples were kept in sample bottles and multilayer pouches to examine the effect of packaging material on the rate of degradation of sesamolin and its effect on Baudouin test. Similarly, addition of antioxidant (0.02% BHA) was carried out to evaluate the effect of antioxidant on the degradation of sesamolin.

4.2.1 Changes in Baudouin test during storage of adulterated ghee samples at 37ºC for 8 months

Fresh and adulterated ghee samples were subjected to Baudouin test and results are depicted in Fig 4.14 and 4.15, respectively. Findings from the present work revealed that Baudouin test remained positive in adulterated ghee samples with higher concentrations of vanaspati (i.e. 10, 20 and 30%), whereas at lower concentrations of vanaspati (1 and 5%) negative results were obtained. It can be inferred from Fig 4.16 to 4.19 that Baudouin test confirmed the presence of sesamolin throughout the storage period. Similarly positive results were observed for the samples stored in sample bottles and multilayer pouches respectively, which indicated that packaging material had no or negligible effect on Baudouin test. No changes were observed in results for Baudouin test for samples with and without BHA. Hence, it can be concluded that storage had no adverse effect on Baudouin test.

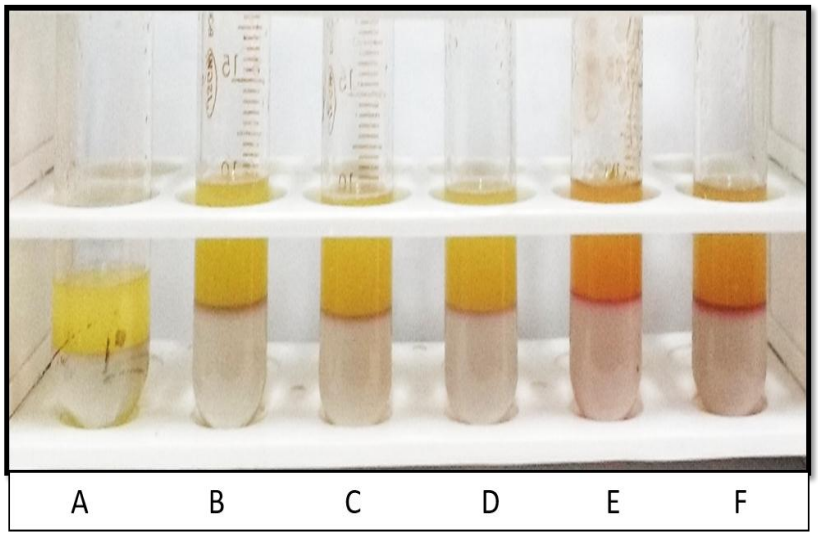


Fig 4.14 Baudouin test for adulterated ghee samples (with BHA) on 0 month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

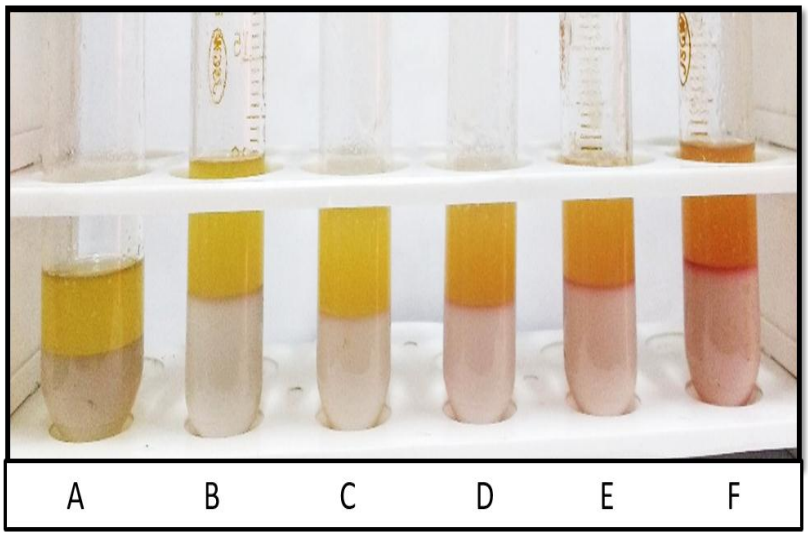


Fig 4.15 Baudouin test for adulterated ghee samples (without BHA) on 0 month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

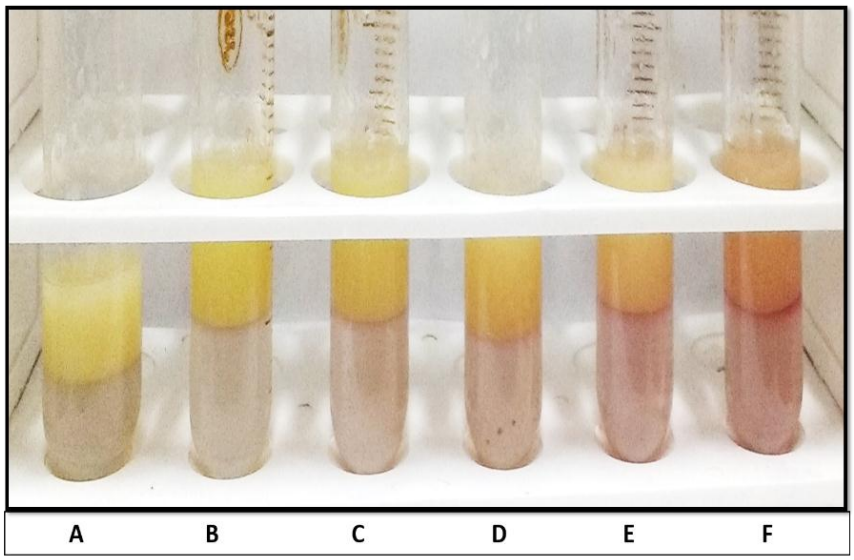


Fig 4.16 Baudouin test for adulterated ghee samples (with BHA) stored in sample bottle on 8th month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

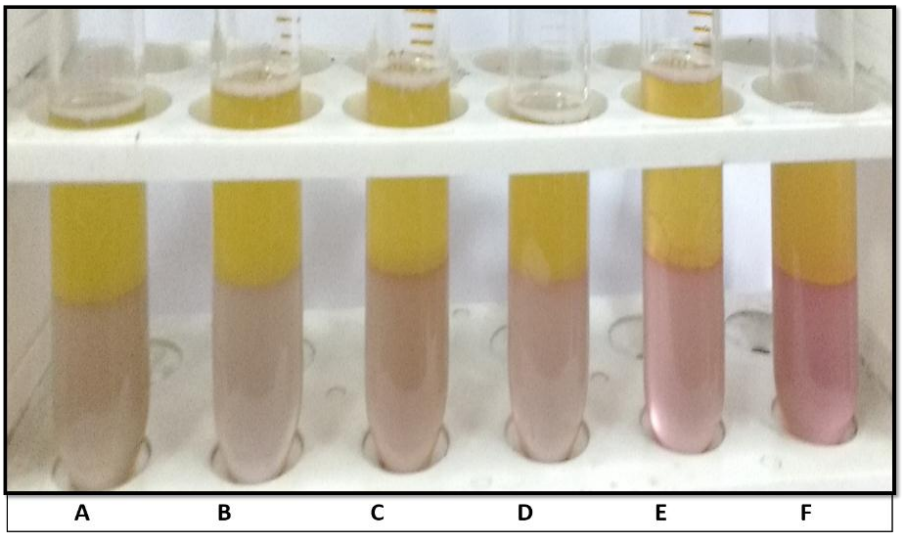


Fig 4.17 Baudouin test for adulterated ghee samples (without BHA) stored in sample bottle on 8th month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

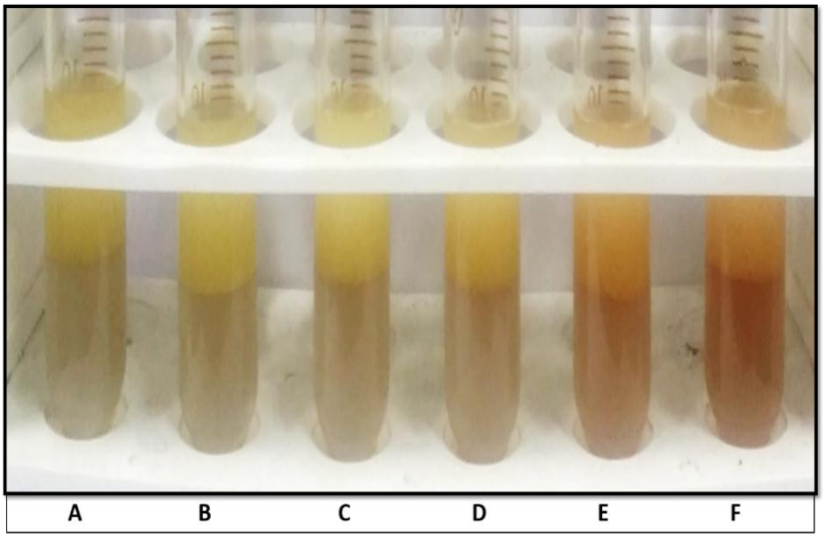


Fig 4.18 Baudouin test for adulterated ghee samples (with BHA) stored in multilayer pouch on 8th month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

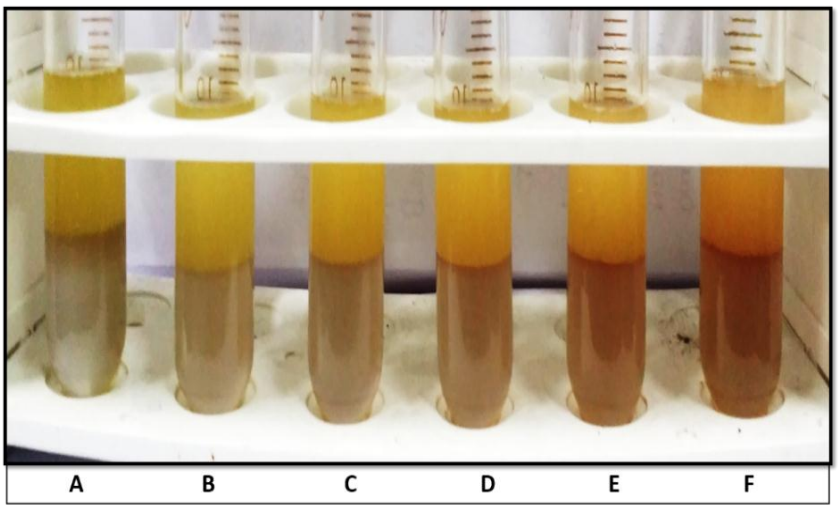


Fig 4.19 Baudouin test for adulterated ghee samples (without BHA) stored in multilayer pouch on 8th month of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

4.2.2 Effect of accelerated storage (80ºC for 10 days) on Baudouin test in adulterated ghee samples

Baudouin test was performed on adulterated ghee samples stored under accelerated storage conditions to observe the changes in the content of sesamolin. Adulterated ghee samples were stored at 80ºC for 10 days in glass bottles. On the beginning of the storage period, adulterated ghee samples were subjected to Baudouin test. It was observed from Fig 4.20 and 4.21 that positive results of Baudouin test were obtained in ghee (with and without BHA) adulterated with vanaspati at 10% and higher concentrations (20% and 30%), whereas, at lower concentrations (1% and 5%) negative results were observed. Results obtained during the storage period were similar to the results obtained on initial day of storage. Fig 4.22.and 4.23 represented the results obtained on 10^{th} day of storage.

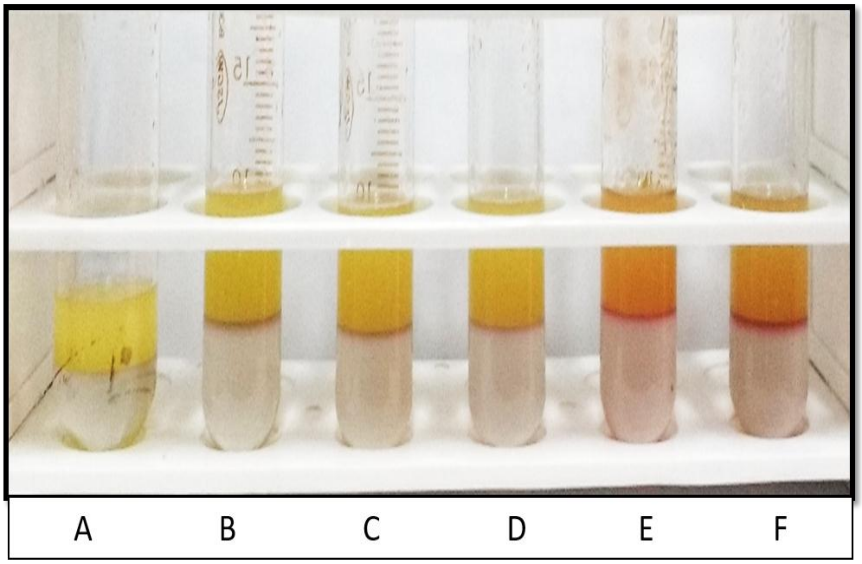


Fig 4.20 Baudouin test for adulterated ghee samples (with BHA) on 0 day of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

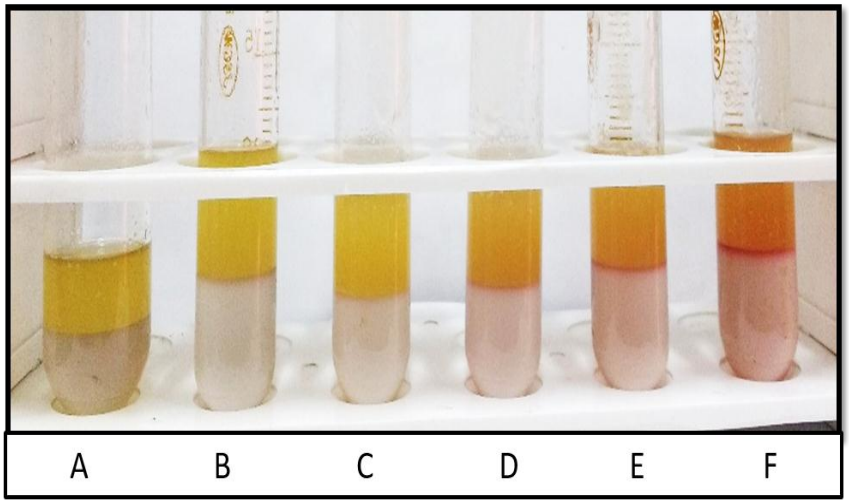


Fig 4.21 Baudouin test for adulterated ghee samples (without BHA) on 0 day of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

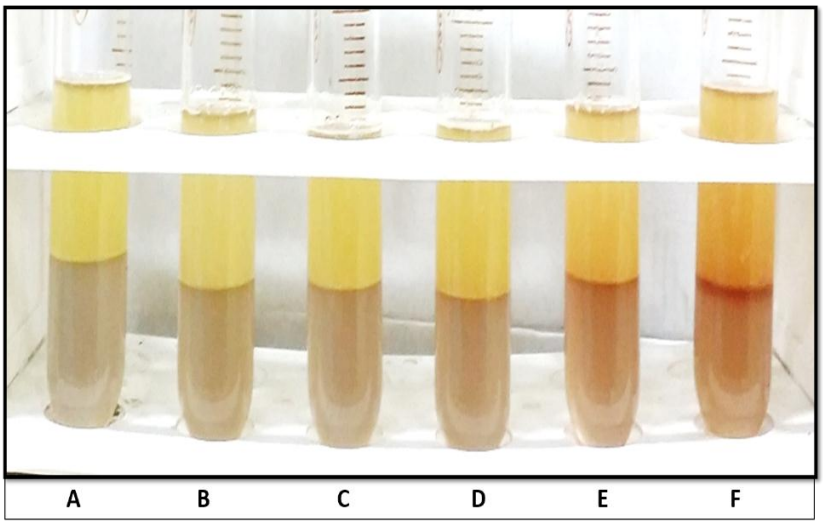


Fig 4.22 Baudouin test for adulterated ghee samples (without BHA) on 10th day of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

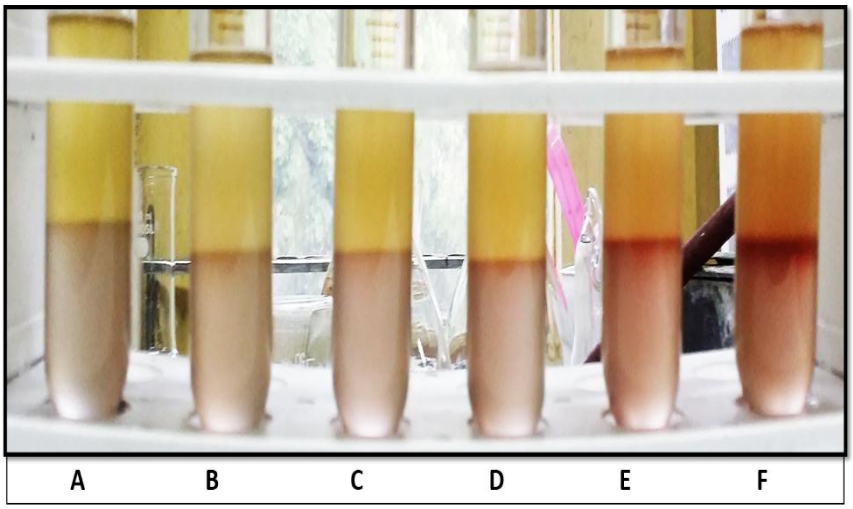


Fig 4.23 Baudouin test for adulterated ghee samples (with BHA) on 10th day of storage

A) Pure ghee, B) 1% adulterated, C) 5% adulterated, D) 10% adulterated, E) 20% adulterated, F) 30% adulterated

4.3 Changes in peroxide value during storage

Peroxide value is the measure of hydroperoxides formed during autoxidation. Rao and Ramamurthy (1987) reported simultaneous formation and breakdown of peroxides leading to accumulation of monocarbonyls during lipid oxidation. Peroxide value represents primary reaction products of lipid oxidation, which can be measured by their ability to liberate iodine from potassium iodide. It is considered to represent the quantity of active oxygen contained in 1 g of lipid. In the present work, adulterated ghee samples (with and without BHA) were stored at 37ºC for 8 months and 80ºC for 10 days. Peroxide value was estimated in all the samples at an interval of 2 months and 2 days during storage for 8 months and 10 days, respectively.

4.3.1 Changes in the peroxide value of adulterated ghee samples (with and without BHA) during storage at 37ºC for 8 months

The changes in peroxide value of pure ghee along with adulterated ghee samples (with and without BHA) are presented in table 4.1 to 4.6 and Fig 4.24 to 4.35, respectively. To analyze the effect of packaging material on peroxide

value, samples were kept in two different type of packaging materials (sample bottles and multilayer pouches) and stored at 37ºC for 8 months.

Significant difference (P<0.05) was observed in peroxide value of pure ghee samples and adulterated ghee samples during storage at 37ºC for 8 months. Addition of increasing concentration of vanaspati (i.e. 1 to 30%) to the pure ghee resulted in higher peroxide value. All the ghee samples showed non-significant (P<0.05) difference on 0^{th} day of storage. It was observed that ghee (without BHA) showed significantly higher (P<0.05) peroxide value than ghee (with BHA) due to absence of antioxidant during storage at 37ºC for 8 months. It was also observed that ghee stored in multilayer pouches showed significantly higher (P<0.05) peroxide values than ghee stored in sample bottles.

Table 4.1 Changes in the peroxide value of pure ghee (with and without BHA) during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of pure ghee				
Storage (Months)	Sample bottle		Multilayer pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.02 ± 0.016^{aA}	0.02 ± 0.016^{aA}	0.02 ± 0.016^{aA}	0.02 ± 0.016^{aA}
2	0.11 ± 0.017^{bB}	0.02 ± 0.015^{aA}	0.13 ± 0.017^{bB}	0.02 ± 0.017^{aA}
4	0.83 ± 0.015^{bC}	0.75 ± 0.028^{aB}	0.85 ± 0.029^{bC}	0.78 ± 0.017^{aB}
6	1.27 ± 0.034^{cD}	0.87 ± 0.044^{aC}	1.37 ± 0.017^{dD}	1.02 ± 0.015^{bC}
8	2.00 ± 0.029^{cE}	1.53 ± 0.034^{aD}	2.10 ± 0.057^{dE}	1.65 ± 0.050^{bD}

Data are presented as means±SEM (n=3)

[a-d]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other

[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

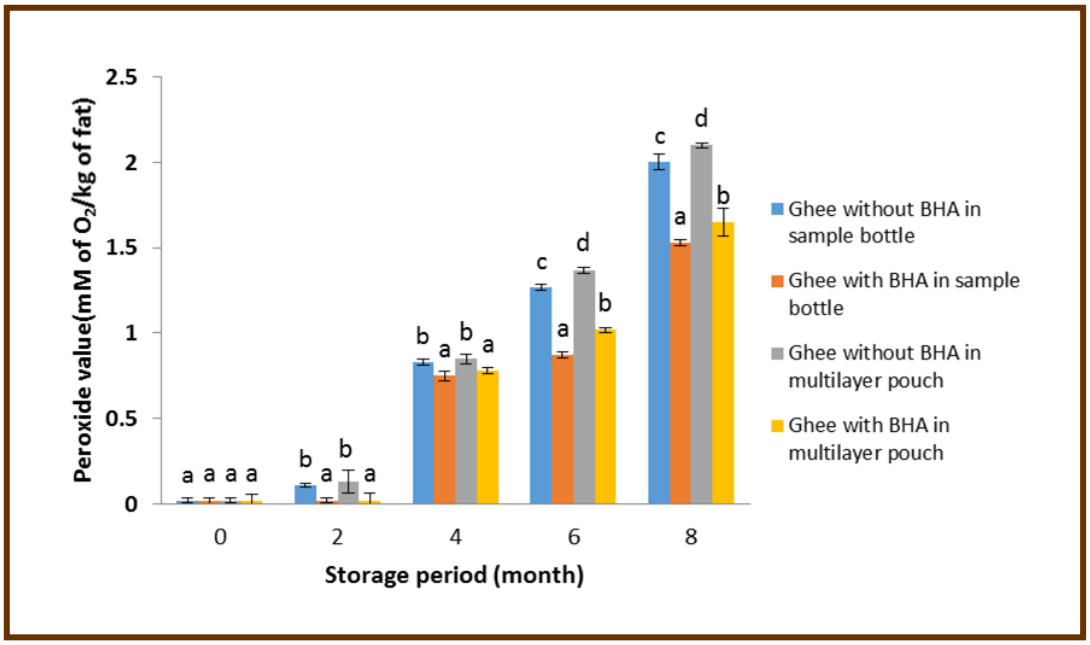


Fig 4.24 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of pure ghee stored at 37ºC for 8 months

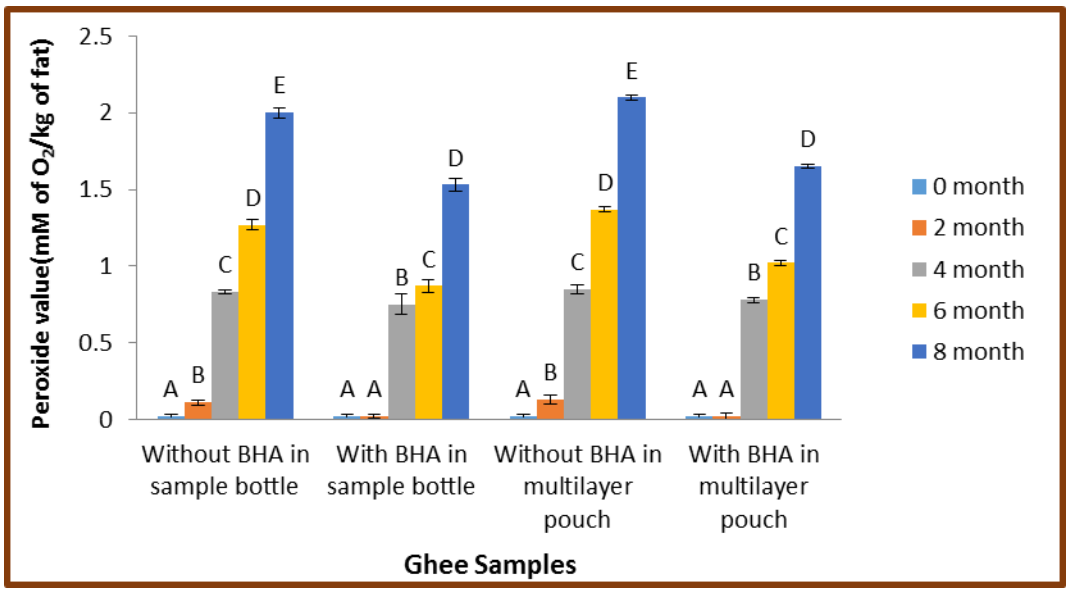


Fig 4.25 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of pure ghee

Our results were in accordance with the findings of Gosewade (2013) who reported significant increase in peroxide values of ghee samples without BHA in comparison with BHA added ghee samples during storage at 37ºC for 8 months. Bunitinx *et al.* (2014) reported that with the increasing surface area and thinning of packaging material, oxygen transmission rate also increased. It can therefore be concluded that higher peroxide value was observed in multilayer pouches which could be due lower thickness and higher surface area of multilayer pouch than sample bottle which affected the oxygen transmission rate during storage.

Table 4.2 Changes in the peroxide value of ghee (with and without BHA) adulterated with 1% vanaspati during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati				
Storage (Months)	Sample bottle		Multilayer Pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.10 ± 0.001^{aA}	0.08 ± 0.013^{aA}	0.10 ± 0.001^{aA}	0.08 ± 0.013^{aA}
2	0.17 ± 0.017^{bB}	0.08 ± 0.001^{aA}	0.27 ± 0.034^{cB}	0.10 ± 0.017^{aA}
4	1.07 ± 0.017^{bC}	0.98 ± 0.034^{aB}	1.08 ± 0.010^{bC}	0.99 ± 0.011^{aB}
6	1.63 ± 0.010^{bD}	1.05 ± 0.029^{aB}	1.77 ± 0.031^{cD}	1.08 ± 0.011^{aB}
8	2.20 ± 0.050^{cE}	1.87 ± 0.034^{aC}	2.30 ± 0.004^{dE}	1.88 ± 0.001^{bC}

Data are presented as means±SEM (n=3)
$^{a-d}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

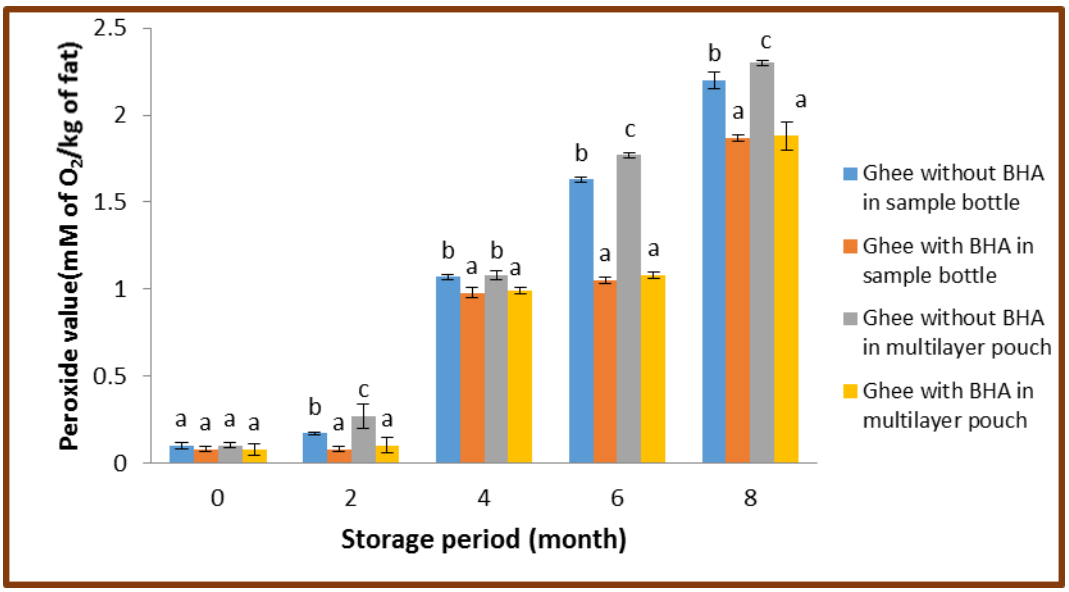


Fig 4.26 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati stored at 37°C for 8 months

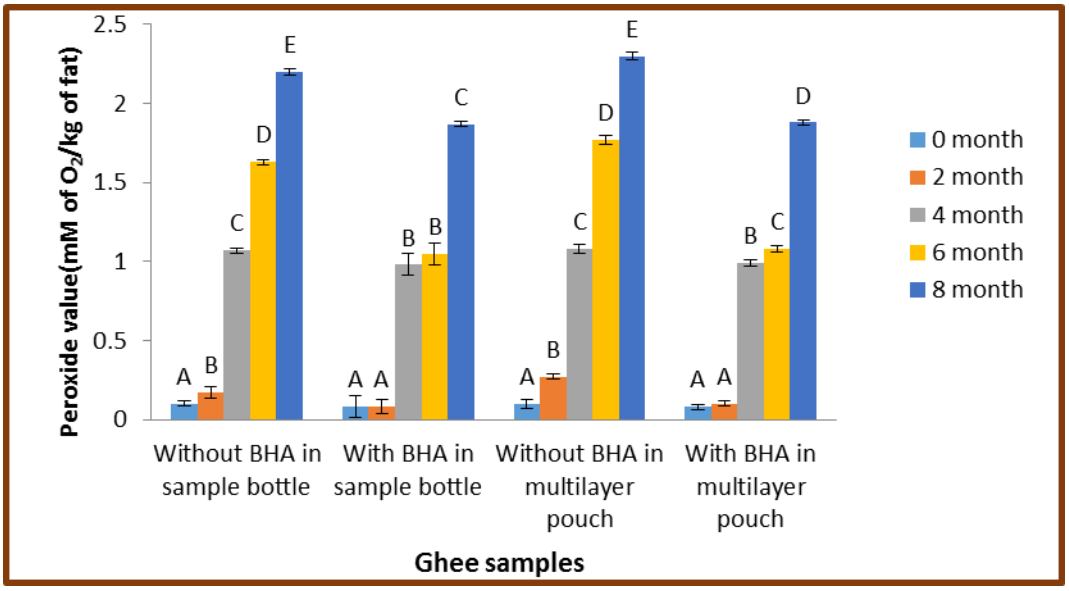


Fig 4.27 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati

Table 4.3 Changes in the peroxide value of ghee (with and without BHA) adulterated with 5% vanaspati during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati				
Storage (Months)	Sample bottle		Multilayer Pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.22 ± 0.019^{aA}	0.22 ± 0.019^{aA}	0.22 ± 0.019^{aA}	0.22 ± 0.019^{aA}
2	0.38 ± 0.016^{bB}	0.27 ± 0.015^{aA}	0.38 ± 0.011^{bB}	0.28 ± 0.033^{aB}
4	1.13 ± 0.001^{cC}	1.05 ± 0.001^{aB}	1.18 ± 0.011^{cC}	1.12 ± 0.017^{bC}
6	1.80 ± 0.029^{cD}	1.12 ± 0.011^{aC}	2.02 ± 0.010^{dD}	1.43 ± 0.005^{bD}
8	2.35 ± 0.028^{cE}	1.78 ± 0.045^{aD}	2.37 ± 0.016^{cE}	1.92 ± 0.034^{bE}

Data are presented as means±SEM (n=3)
[a-d]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

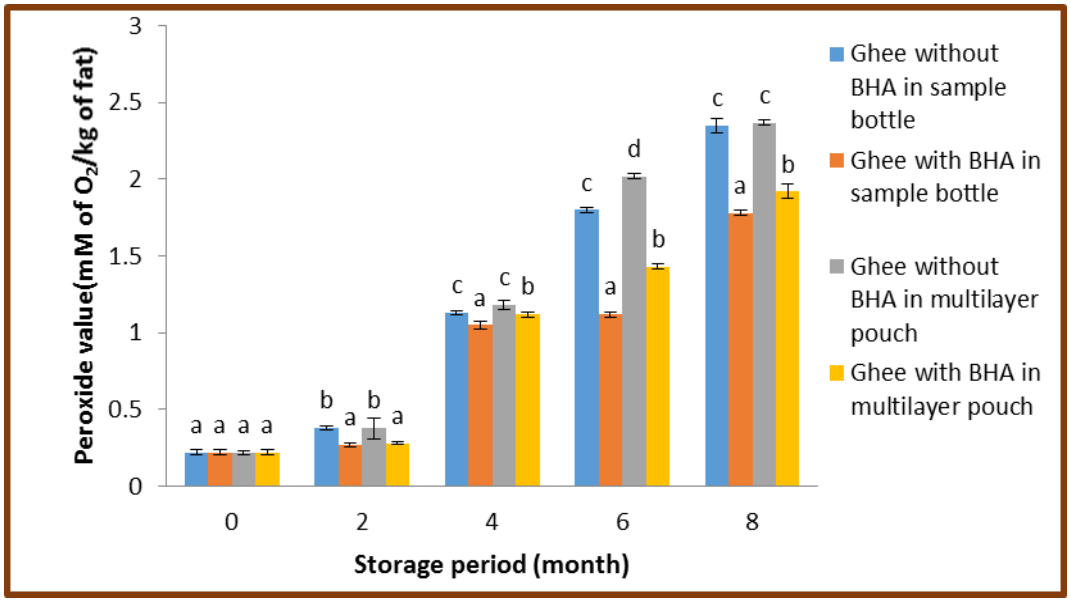


Fig 4.28 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati stored at 37ºC for 8 months

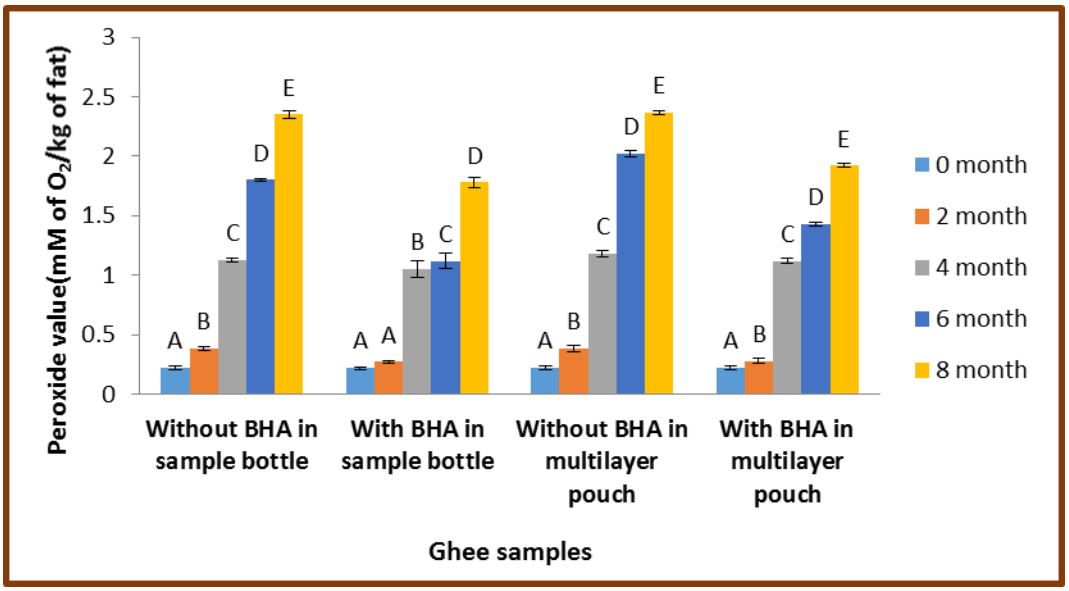


Fig 4.29 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati

Table 4.4 Changes in the peroxide value of ghee (with and without BHA) adulterated with 10% vanaspati during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati				
Storage (Months)	Sample bottle		Multilayer Pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.30 ± 0.033^{aA}	0.31 ± 0.049^{aA}	0.30 ± 0.033^{aA}	0.31 ± 0.049^{aA}
2	1.35 ± 0.017^{cB}	0.73 ± 0.017^{aB}	1.37 ± 0.017^{cB}	1.13 ± 0.017^{bB}
4	2.05 ± 0.072^{cC}	1.47 ± 0.010^{aC}	2.33 ± 0.014^{dC}	1.63 ± 0.012^{bC}
6	2.93 ± 0.034^{cD}	1.77 ± 0.010^{aD}	2.97 ± 0.002^{cD}	2.30 ± 0.032^{bD}
8	3.60 ± 0.034^{cE}	3.03 ± 0.007^{aE}	3.70 ± 0.020^{dE}	3.28 ± 0.009^{bE}

Data are presented as means±SEM (n=3)
$^{a-d}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

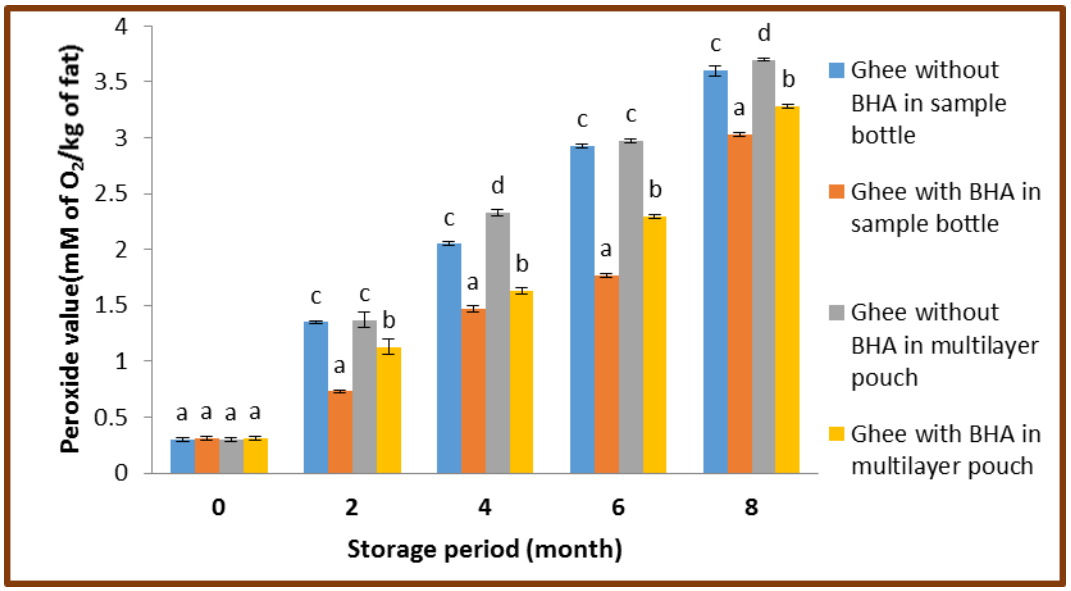


Fig 4.30 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati stored at 37ºC for 8 months

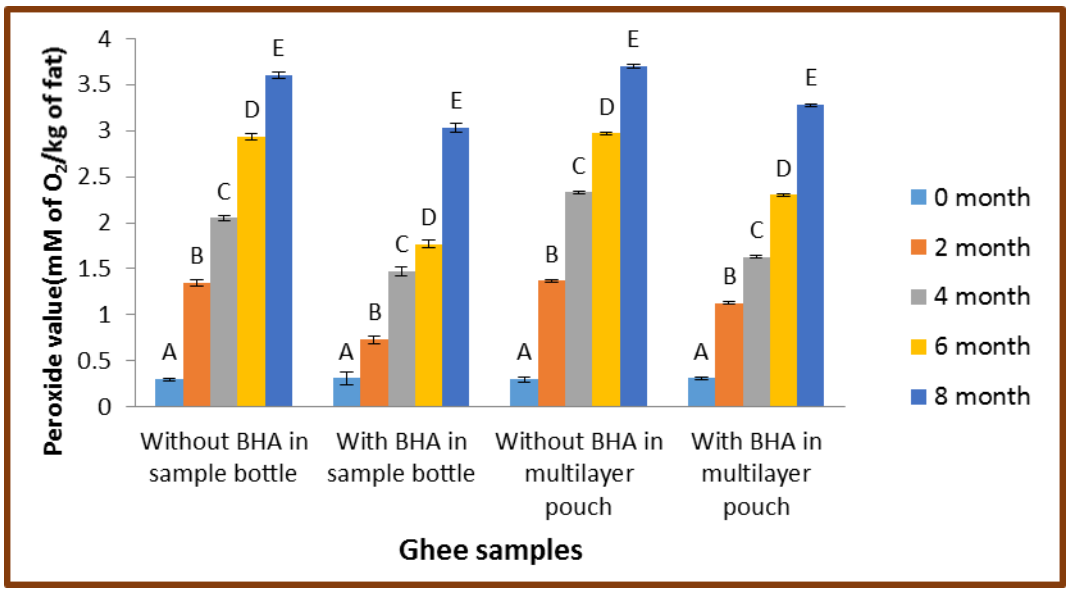


Fig 4.31 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati

Table 4.5 Changes in the peroxide value of ghee (with and without BHA) adulterated with 20% vanaspati during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati				
Storage (Months)	Sample bottle		Multilayer Pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.40 ± 0.029^{aA}	0.42 ± 0.015^{aA}	0.40 ± 0.029^{aA}	0.42 ± 0.015^{aA}
2	2.12 ± 0.012^{cB}	1.67 ± 0.015^{aB}	2.20 ± 0.016^{dB}	1.75 ± 0.017^{bB}
4	2.35 ± 0.027^{cC}	1.71 ± 0.010^{aB}	2.63 ± 0.043^{dC}	1.78 ± 0.021^{bB}
6	3.93 ± 0.016^{cD}	2.97 ± 0.015^{aC}	4.10 ± 0.011^{dD}	3.07 ± 0.029^{bC}
8	4.63 ± 0.025^{cE}	4.28 ± 0.001^{aD}	4.67 ± 0.010^{cE}	4.33 ± 0.003^{bD}

Data are presented as means±SEM (n=3)
[a-d]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

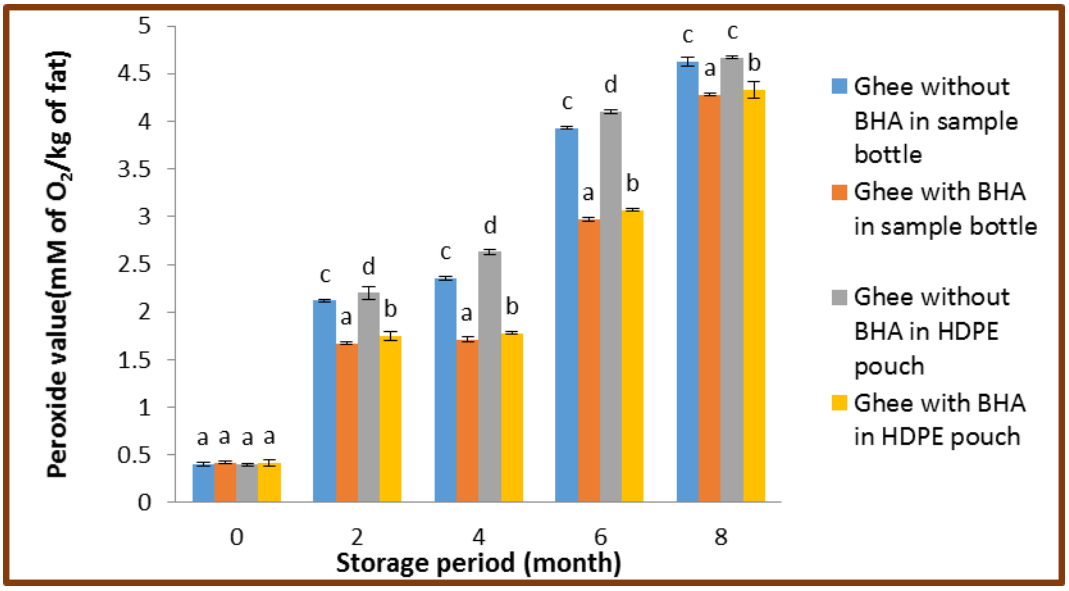


Fig 4.32 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati stored at 37ºC for 8 months

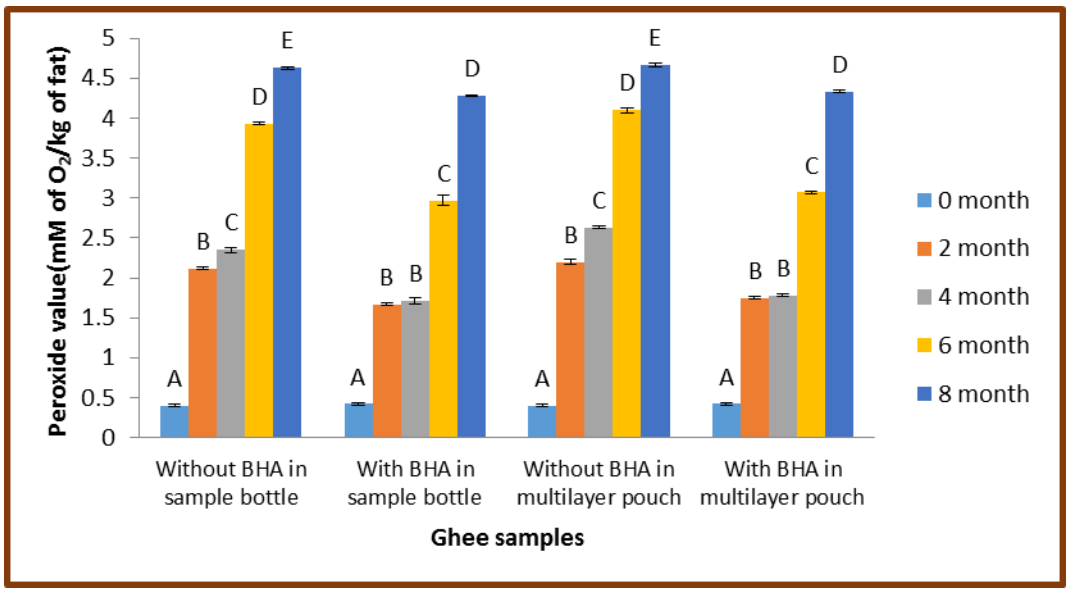


Fig 4.33 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati

Table 4.6 Change in the peroxide value of ghee (with and without BHA) adulterated with 30% vanaspati during storage at 37ºC for 8 months in different packaging materials

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 30% vanaspati				
Storage (Months)	Sample bottle		Multilayer Pouch	
	Ghee (without BHA)	Ghee (with BHA)	Ghee (without BHA)	Ghee (with BHA)
0	0.60 ± 0.057^{aA}	0.57 ± 0.030^{aA}	0.60 ± 0.057^{aA}	0.57 ± 0.030^{aA}
2	2.75 ± 0.017^{cB}	1.85 ± 0.013^{aB}	2.93 ± 0.011^{dB}	1.92 ± 0.010^{bB}
4	2.97 ± 0.014^{cC}	2.63 ± 0.037^{aC}	3.03 ± 0.017^{dC}	2.78 ± 0.007^{bC}
6	4.35 ± 0.021^{cD}	3.63 ± 0.025^{aD}	4.38 ± 0.009^{cD}	3.97 ± 0.067^{bD}
8	5.05 ± 0.001^{cE}	4.67 ± 0.035^{aE}	5.08 ± 0.051^{cE}	4.77 ± 0.004^{bE}

Data are presented as means±SEM (n=3)
$^{a-d}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

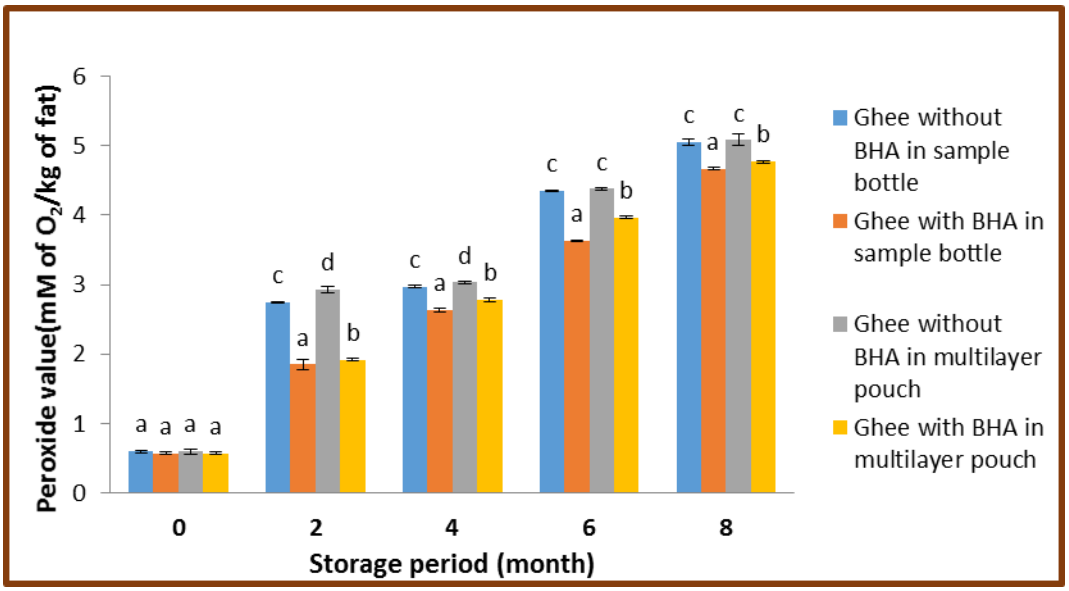


Fig 4.34 Effect of BHA and packaging material on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 30% vanaspati stored at 37°C for 8 months

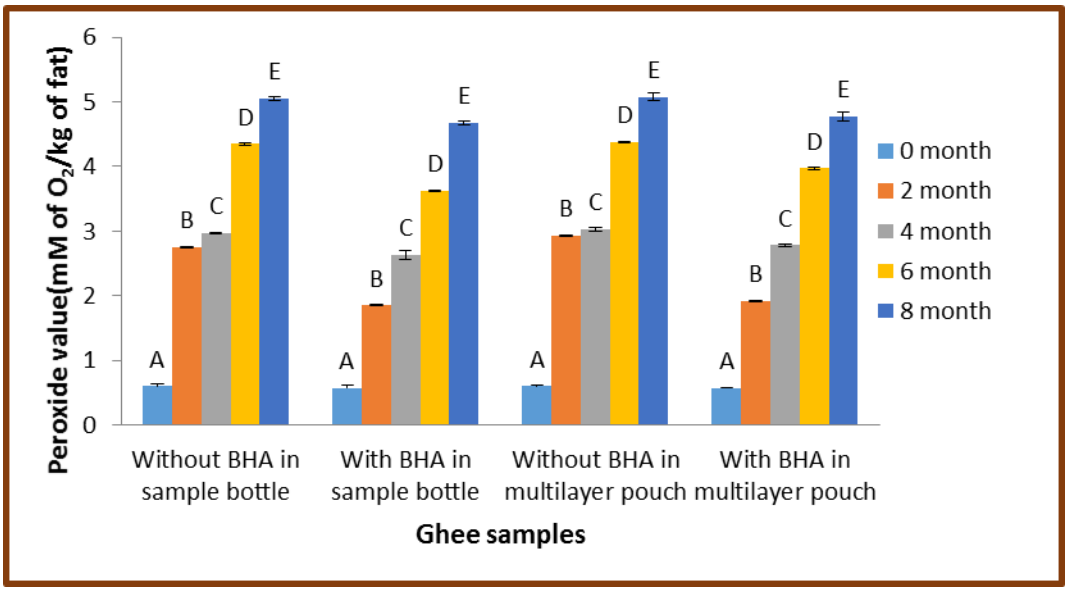


Fig 4.35 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 30% vanaspati

4.3.2 Changes in the peroxide value (millimoles of O_2 per kg fat) of ghee samples adulterated with vanaspati during storage at 80ºC for 10 days (Accelerated storage).

Peroxide value was also estimated in ghee samples adulterated with vanaspati during storage in glass bottles under accelerated storage conditions at 80ºC for 10 days. Results of peroxide value of all the ghee samples are presented in Table 4.7 to 4.12 as well as in Fig 4.36 to 4.47, respectively. It was evident from the results that all the ghee samples differed from each other significantly ($P<0.05$) in peroxide value. It was observed that ghee (without BHA) showed significantly higher ($P<0.05$) peroxide value than ghee samples with BHA. Lower peroxide value was mainly attributed due to presence of antioxidant which prevented the oxidation of fat and lowered the formation of peroxides. Similar trend was observed in all adulterated ghee samples throughout the storage period. Results of the present investigation were in accordance with the findings of Pawar *et al*. (2014), who reported higher peroxide in ghee without BHA than ghee with BHA after 21 days at 80 ± 1 °C

Table 4.7 Changes in the peroxide value of pure ghee (with and without BHA) during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of pure ghee		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.02 ± 0.017^{aA}	0.02 ± 0.016^{aA}
2	0.32 ± 0.016^{bB}	0.13 ± 0.017^{aB}
4	0.85 ± 0.028^{bC}	0.32 ± 0.017^{aC}
6	1.17 ± 0.034^{bD}	0.93 ± 0.034^{aD}
8	2.06 ± 0.014^{bE}	1.76 ± 0.001^{aE}
10	2.67 ± 0.034^{bF}	2.05 ± 0.021^{aF}

Data are presented as means±SEM (n=3)

[a-b]Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other

[A-F]Means within column with different uppercase superscript are significantly different ($P<0.05$) from each other

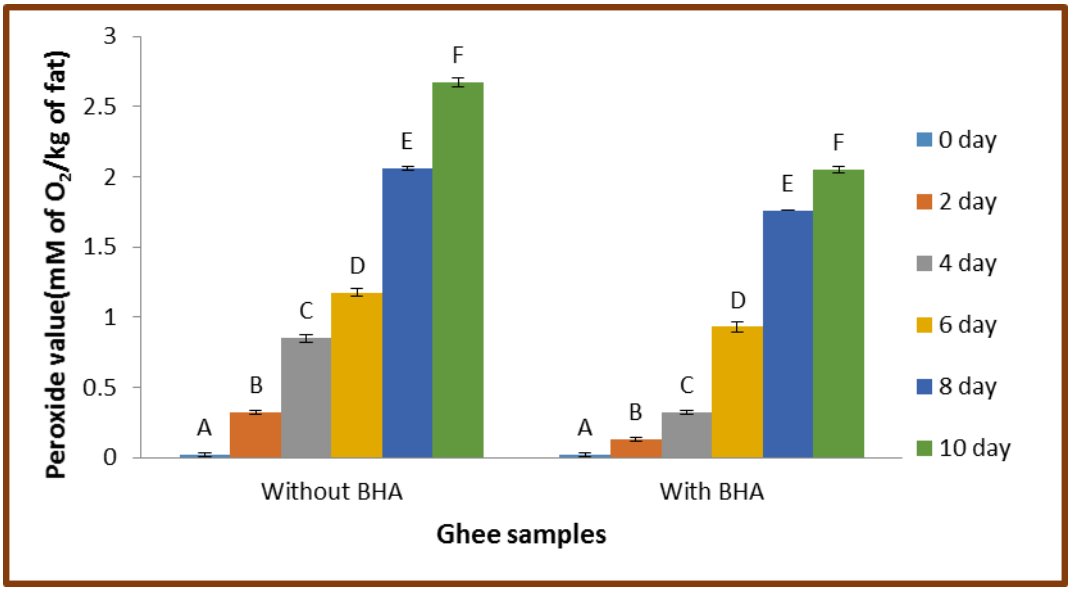


Fig 4.36 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of pure ghee stored at 80ºC for 10 days

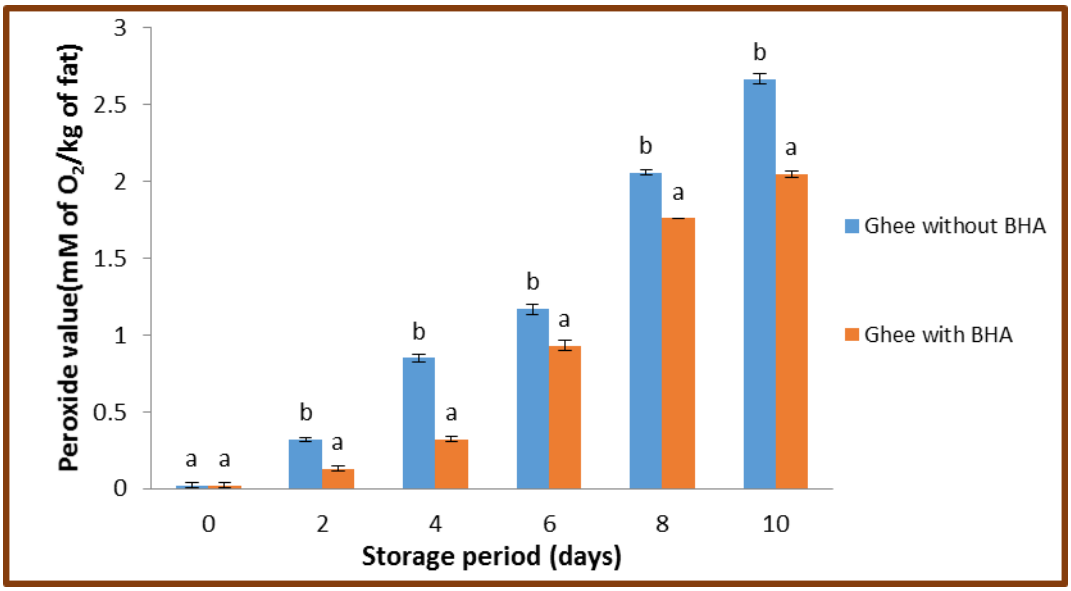


Fig 4.37 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of pure ghee

Table 4.8 Changes in the peroxide value of ghee (with and without BHA) adulterated with 1% vanaspati during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.10 ± 0.001^{aA}	0.08 ± 0.013^{aA}
2	0.53 ± 0.032^{bB}	0.23 ± 0.011^{aB}
4	0.90 ± 0.001^{bC}	0.57 ± 0.067^{aC}
6	1.35 ± 0.028^{bD}	1.12 ± 0.017^{aD}
8	2.24 ± 0.028^{bE}	1.88 ± 0.017^{aE}
10	2.95 ± 0.029^{bF}	2.25 ± 0.024^{aF}

Data are presented as means±SEM (n=3)
[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

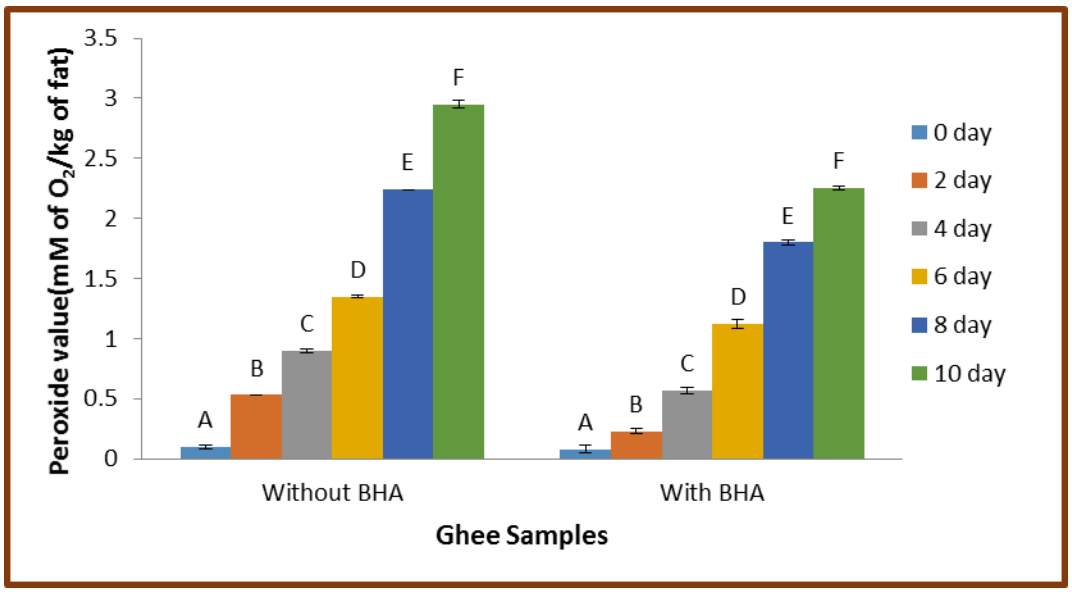


Fig 4.38 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati stored at 80ºC for 10 days

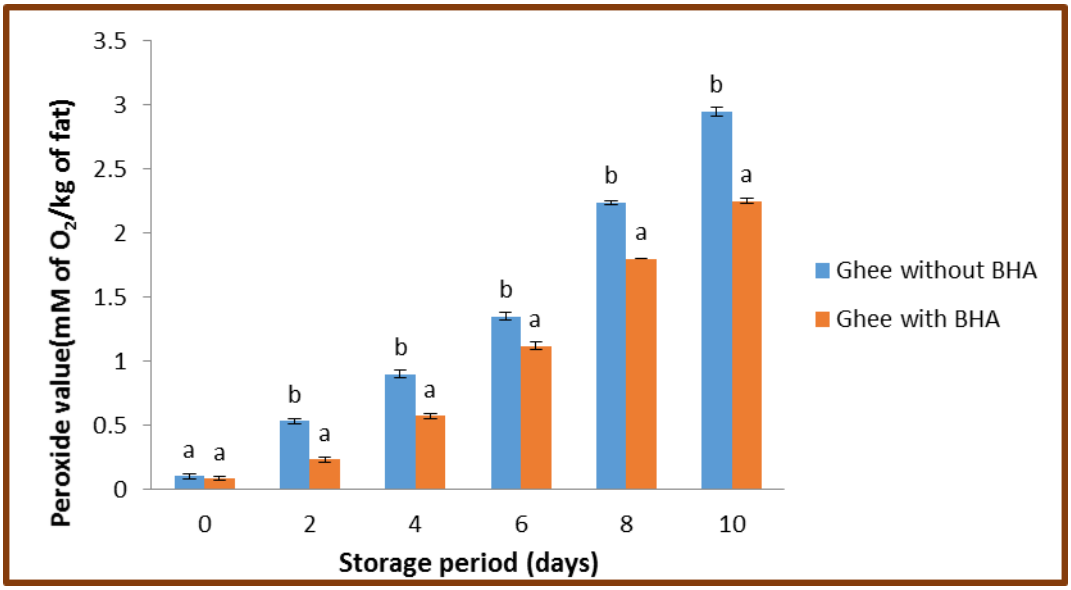


Fig 4.39 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 1% vanaspati

Table 4.9 Changes in the peroxide value of ghee (with and without BHA) adulterated with 5% vanaspati during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.22 ± 0.019^{aA}	0.22 ± 0.012^{aA}
2	0.63 ± 0.010^{bB}	0.35 ± 0.028^{aB}
4	1.32 ± 0.017^{bC}	1.05 ± 0.029^{aC}
6	1.73 ± 0.033^{bD}	1.57 ± 0.034^{aD}
8	2.46 ± 0.033^{bE}	2.12 ± 0.034^{aE}
10	3.33 ± 0.034^{bF}	2.57 ± 0.064^{aF}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

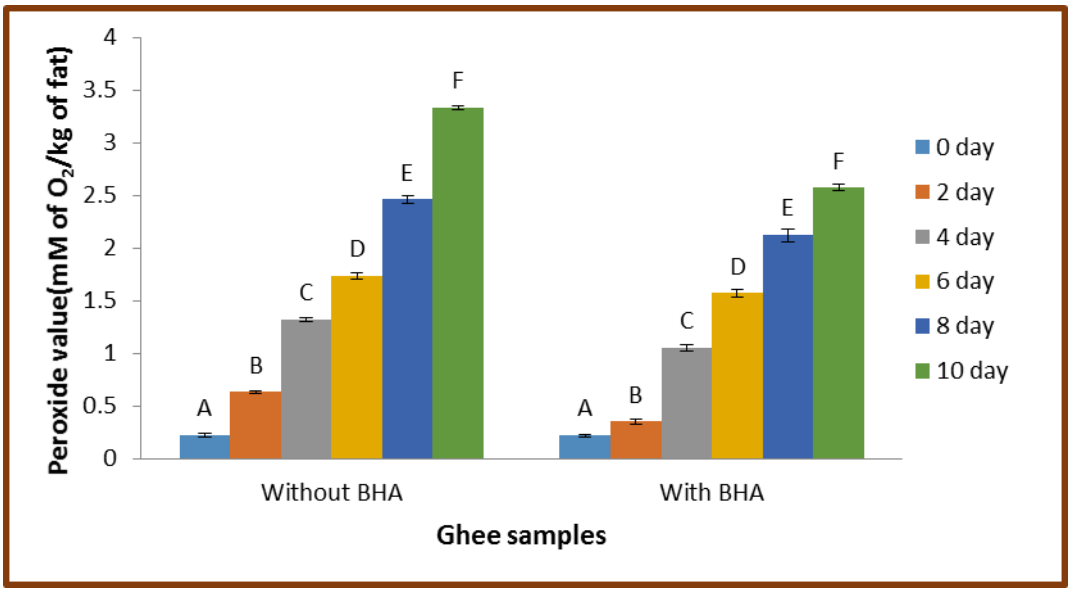


Fig 4.40 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati stored at 80ºC for 10 days

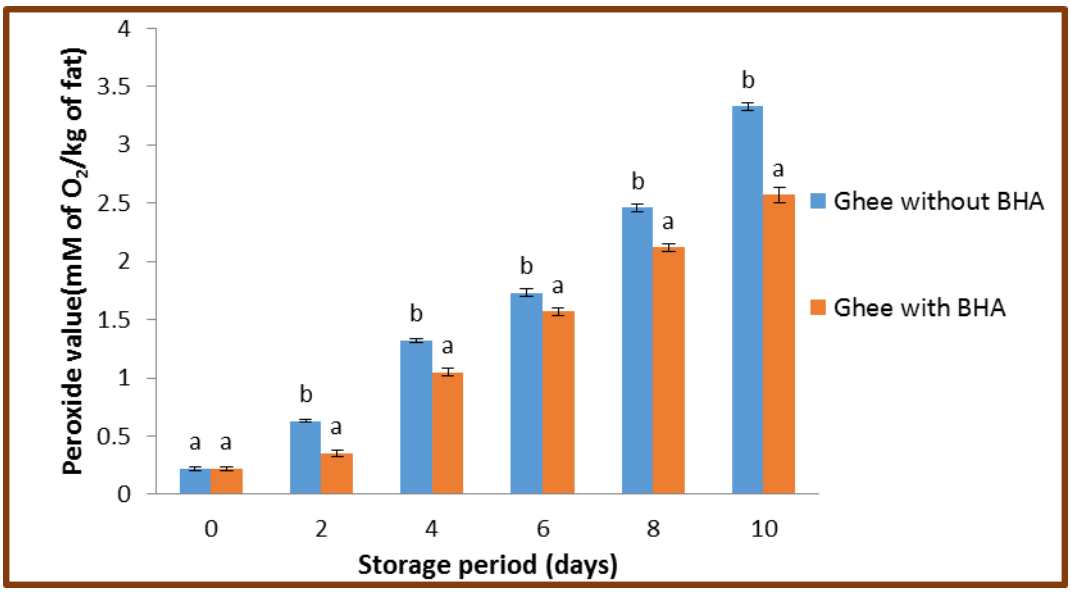


Fig 4.41 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 5% vanaspati

Table 4.10 Changes in the peroxide value of ghee (with and without BHA) adulterated with 10% vanaspati during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.27 ± 0.033^{aA}	0.30 ± 0.049^{aA}
2	0.73 ± 0.009^{bB}	0.53 ± 0.017^{aB}
4	1.37 ± 0.044^{bC}	1.13 ± 0.034^{aC}
6	1.82 ± 0.017^{bD}	1.67 ± 0.031^{aD}
8	2.94 ± 0.010^{bE}	2.54 ± 0.011^{aE}
10	3.98 ± 0.044^{bF}	3.32 ± 0.017^{aF}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

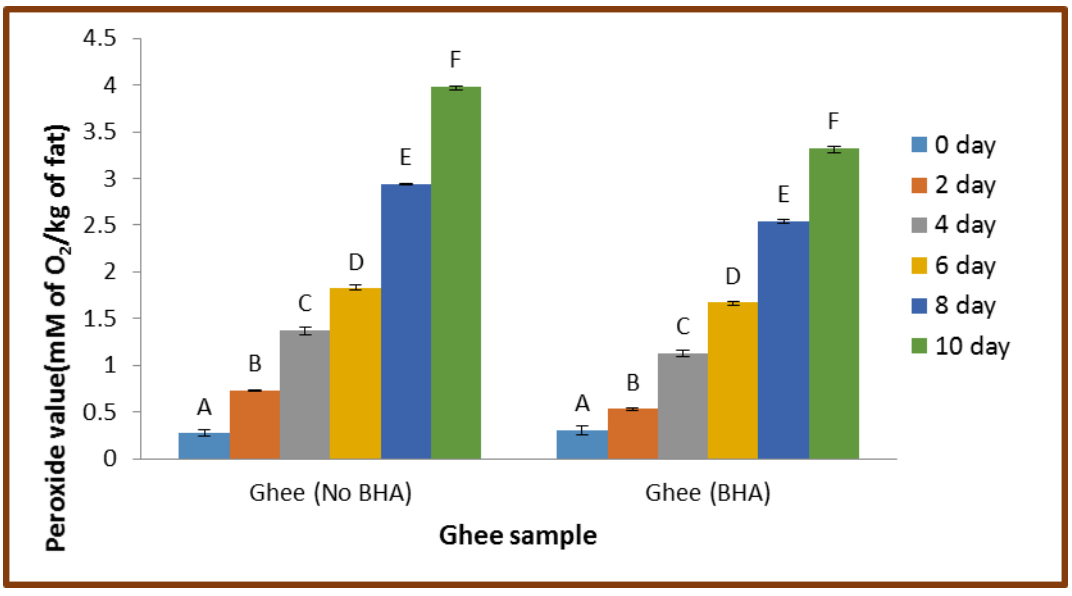


Fig 4.42 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati stored at 80ºC for 10 days

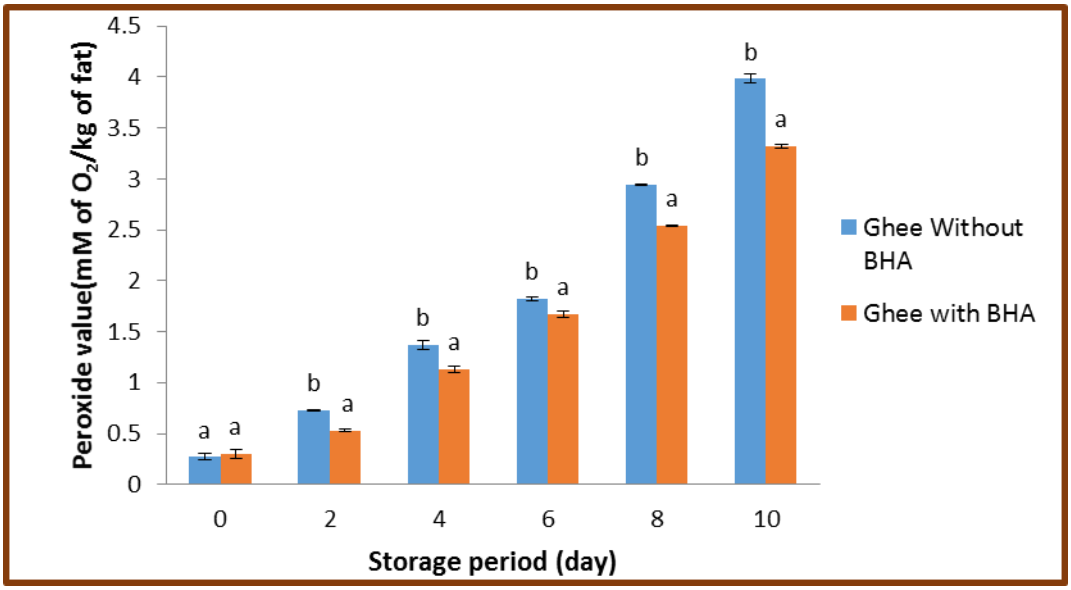


Fig 4.43 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 10% vanaspati

Table 4.11 Changes in the peroxide value of ghee (with and without BHA) adulterated with 20% vanaspati during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.40 ± 0.029^{aA}	0.42 ± 0.015^{aA}
2	1.07 ± 0.012^{bB}	0.68 ± 0.009^{aB}
4	2.28 ± 0.033^{bC}	2.0 ± 0.029^{aC}
6	3.83 ± 0.034^{bD}	3.47 ± 0.026^{aD}
8	4.77 ± 0.034^{bE}	3.84 ± 0.026^{aE}
10	5.45 ± 0.027^{bF}	4.27 ± 0.084^{aF}

Data are presented as means±SEM (n=3)
[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

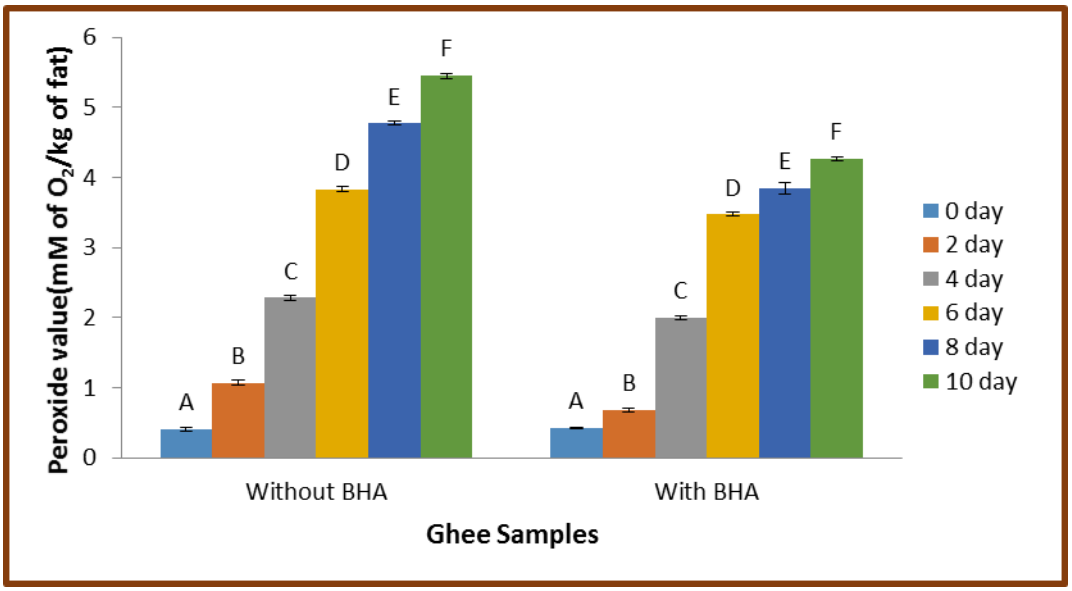


Fig 4.44 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati stored at 80ºC for 10 days

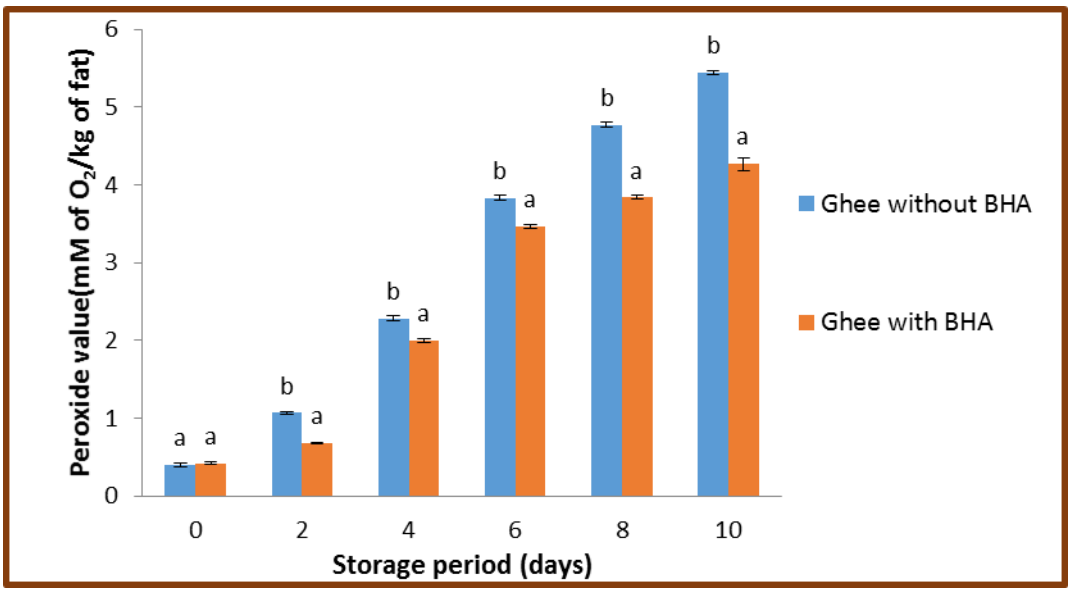


Fig 4.45 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 20% vanaspati

Table 4.12 Changes in the peroxide value of ghee (with and without BHA) adulterated with 30% vanaspati during storage at 80ºC for 10 days

Peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 30% vanaspati		
Storage (days)	Ghee (without BHA)	Ghee (with BHA)
0	0.60 ± 0.057^{aA}	0.57 ± 0.030^{aA}
2	1.62 ± 0.012^{bB}	1.33 ± 0.016^{aB}
4	2.72 ± 0.016^{bC}	2.43 ± 0.065^{aC}
6	4.03 ± 0.030^{bD}	3.63 ± 0.021^{aD}
8	5.27 ± 0.030^{bE}	4.34 ± 0.011^{aE}
10	6.02 ± 0.010^{bF}	4.80 ± 0.057^{aF}

Data are presented as means±SEM (n=3)
[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

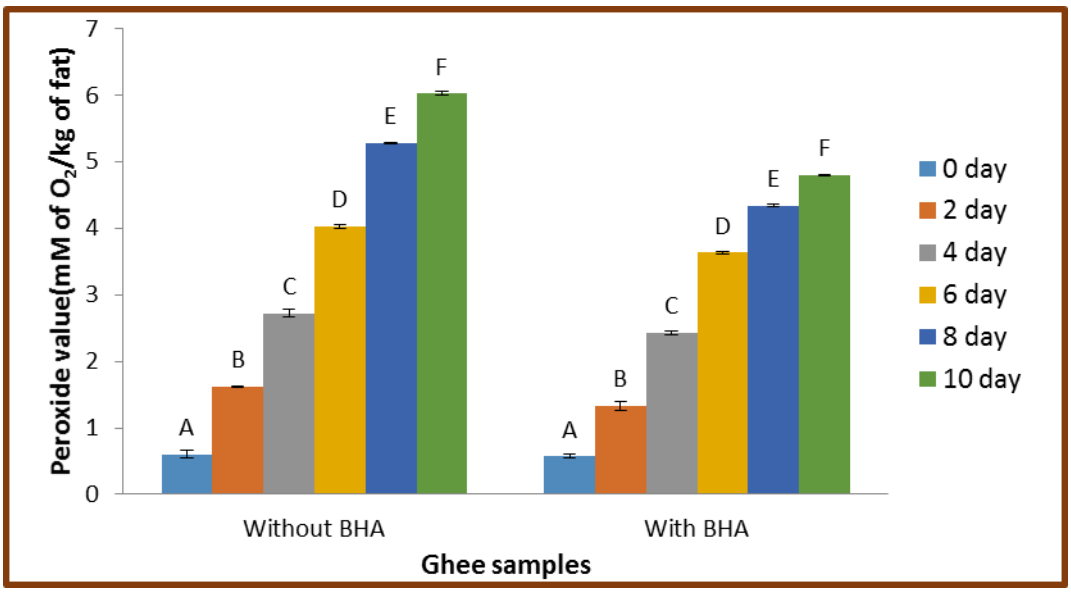


Fig 4.46 Effect of BHA on peroxide value (millimoles of O_2 per kg fat) of 30% vanaspati adulterated ghee stored at 80ºC for 10 days

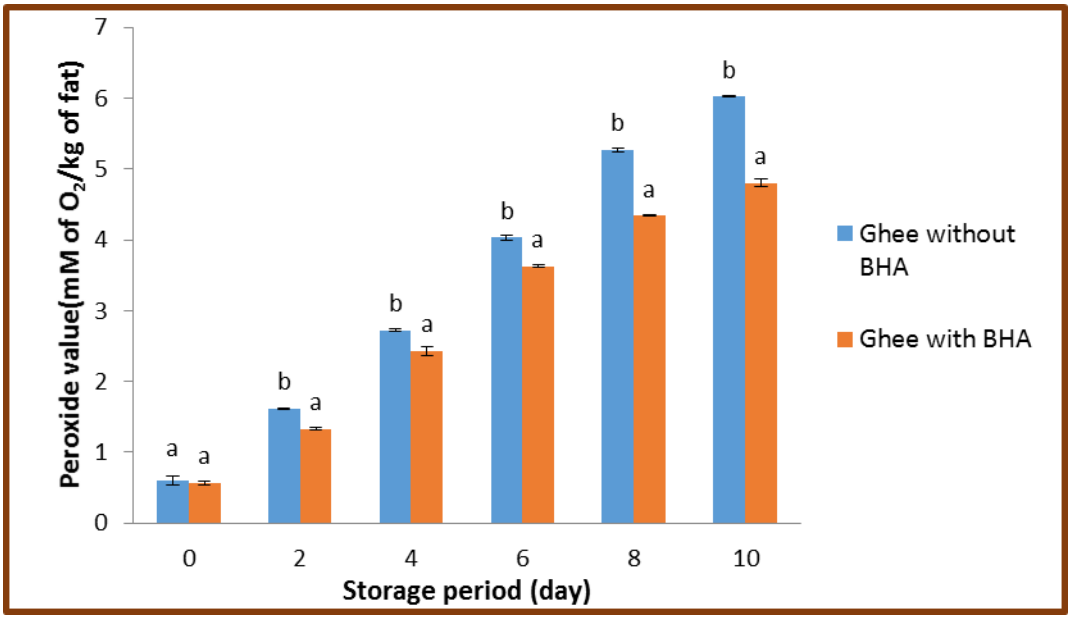


Fig 4.47 Effect of storage on peroxide value (millimoles of O_2 per kg fat) of ghee adulterated with 30% vanaspati

Results of the peroxide value measurement clearly showed that storage led to an increase in peroxide value of ghee samples. However, the Sesamin and Baudouin tests remained unaffected as a result of storage. This clearly demonstrated that the indicator compounds i.e. sesamin and sesamolin were quite stable and were not affected by oxidation of fat. Hence, both the tests i.e. Sesamin and Baudouin test can be used successfully in stored samples to detect the presence of vanaspati or partially hydrogenated vegetable oil in ghee.

4.4 Profiling of sesamin and sesamolin by chromatographic techniques

Adulterated ghee samples were stored at different time temperature combinations to evaluate the changes in the concentration of sesamin and sesamolin. Effect of storage on these two compounds was evaluated using Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC) and correlated the results with the results of Baudouin and Sesamin test. Adulterated ghee samples stored at 37ºC for 8 months were subjected to TLC for profiling of sesamin and sesamolin whereas, adulterated ghee samples stored at 80ºC for 10 days were subjected to both TLC and HPLC for profiling of sesamin and sesamolin and correlated with the test results obtained for Baudouin and Sesamin test.

4.4.1 Detection of sesamin and sesamolin by Thin Layer Chromatography

Adulterated ghee samples stored at 37ºC for 8 months were subjected to TLC for detection of sesamin and sesamolin.

4.4.1.1 Selection of solvent system to resolve standard sesamol, sesamin and sesamolin for Thin Layer Chromatography

Normal phase TLC was performed using various solvent systems. The solvent systems used are described in Table 4.13

Table 4.13 Solvent systems used for TLC

Sr. No	Solvent system
1	Chloroform: benzene: methanol (60:40:1 v/v/v)
2	Chloroform: diethyl ether (90:10 v/v)
3	Benzene: acetone (97.5:2.5 v/v)
4	Petroleum ether: diethyl ether: acetic acid (70:30:1 v/v/v)

All the above solvent systems were able to separate standards of sesamin and sesamolin but after studying solvent systems, maximum mobility of sesamin and sesamolin was observed in chloroform: benzene: methanol (60:40:1) solvent system which gave optimum separation as well as clear separated bands of both sesamin and sesamolin as evident from their R_f values (Table 4.15 and Fig 4.48) so, solvent system [chloroform: benzene: methanol (60:40:1 v/v/v)] suggested by Kamal-Eldin *et al*. (1994) was used for further analysis.

4.4.1.2 Selection of spraying reagent for TLC

Normal phase TLC was performed using chloroform: benzene: methanol (60:40:1 v/v/v) solvent system. For the visualization of the bands of sesamin and sesamolin, different spraying reagents were used which have been reported in literature (Table 4.14)

Table 4.14 Spraying reagents used for TLC

Sr. No	Spraying reagent
1	Phosphomolybdic acid
2	Anisaldehyde solution made in ethanol and H_2SO_4
3	20% H_2SO_4 made in water
4	20% H_2SO_4 made in methanol

According to Adegbola *et al*. (2008) maximum clear and sharp bands were obtained with anisaldehyde reagent. On spraying anisaldehyde reagent on TLC plates, sharp red coloured band was obtained for sesamolin. However, the only drawback of this spraying reagent was that it resulted in red coloured background of the TLC plate. Red background of TLC plate and red band of analyte (sesamolin) on TLC plate led to misleading results. Hence, the spraying reagent was not selected. Mohamed and Awatif (1998) used 50% H_2SO_4 made in water for the visualization of sesamin and sesamolin. On using 50% H_2SO_4 in water, it was observed that the developed bands of sesamin and sesamolin had damaged appearance. Therefore, concentration of H_2SO_4 was reduced to 20% and diluents used were water and methanol separately. 20% H_2SO_4 made in methanol resulted into clear and sharp bands of sesamin and sesamolin. The visualisation was better with 20% H_2SO_4 made in methanol as compared to 20% H_2SO_4 made in water. Hence this spraying reagent was selected for visualization of sesamin, sesamol and sesamolin on silica Gel 60 F_{254} Plates.

Table 4.15 R_f value of sesame lignans

Sesame Lignan	R_f Value
Sesamol	0.29±0.003
Sesamolin	0.51±0.006
Sesamin	0.60 ±0.010

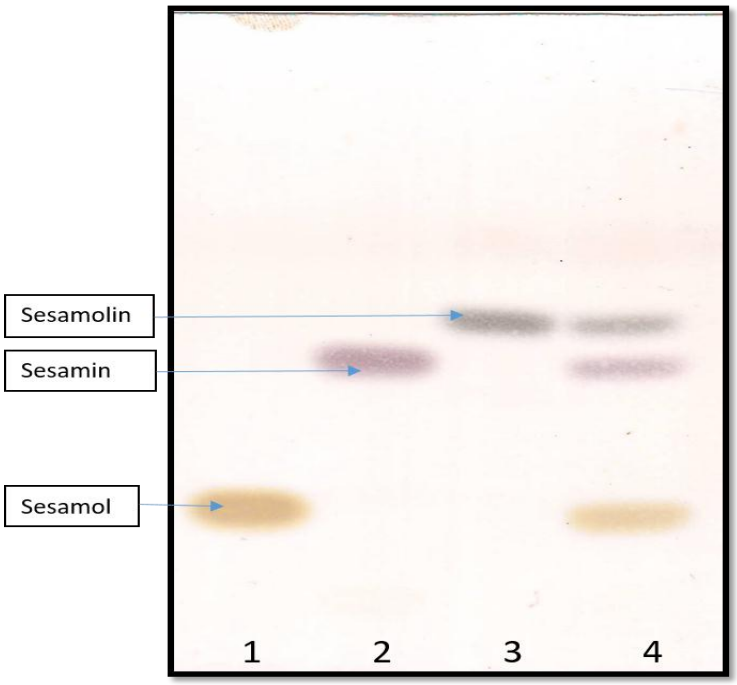


Fig 4.48 Separation of sesamol, sesamin and sesamolin standards on TLC plates

1) Sesamol standard 2) Sesamin standard 3) Sesamolin standard 4) Mixture of sesamol, sesamin and sesamolin standard

4.4.1.3 Isolation of sesamin and sesamolin from ghee

Thin layer chromatography (TLC) analysis was carried out with different solvent systems to detect the presence of sesamin and sesamolin. Initially, two approaches were followed for the isolation of sesamin and sesamolin.

The first approach consisted of isolation of sesamin and sesamolin using solvent extraction procedures and the second approach consisted of extraction of unsaponifiable matter by saponification. Isolation with methanol was carried out by the method of Rangkadilok *et al.* (2010) as well as Schwertner and Rios, (2012). Further, modification in sample size and different solvents (chloroform and hexane) were also tried for extraction but no promising results were obtained. This could be due to the very low content of sesamin and sesamolin present in the adulterated ghee samples that could not be extracted with solvent extraction method. The unsaponifiable matter (USM) from ghee was isolated as per the method standardized by Sharma *et al*. (2009) for smaller quantities of fat (0.1 to 0.2 g). However, the detection of sesamin and sesamolin with this method

was also not possible, which might be due to lower quantity of sesamin and sesamolin present in the USM.

Therefore, for the detection of sesamin and sesamolin by TLC, USM was extracted according to procedure of IS: 3508 (1966) with a higher initial quantity of fat sample (5 g). Further, the saponification of adulterated ghee samples was carried out with 5% potassium hydroxide solution made in methanol and extraction was carried out with diethyl ether.

4.4.1.4 Determination of detection level

Determination of minimum level of sesamin and sesamolin to ascertain adulteration in ghee samples (1 to 5% added vanaspati) was carried out. Unsaponifiable matter was extracted from the ghee samples to obtain sesamin and sesamolin. USM was extracted from both pure ghee and adulterated ghee samples and then dissolved in 500 µl chloroform and 500 µl methanol (1:1) and applied on TLC plates. The results obtained are shown in Fig 4.49. The minimum detection level was 2% on the basis of sesamin and 4% on the basis of sesamolin.

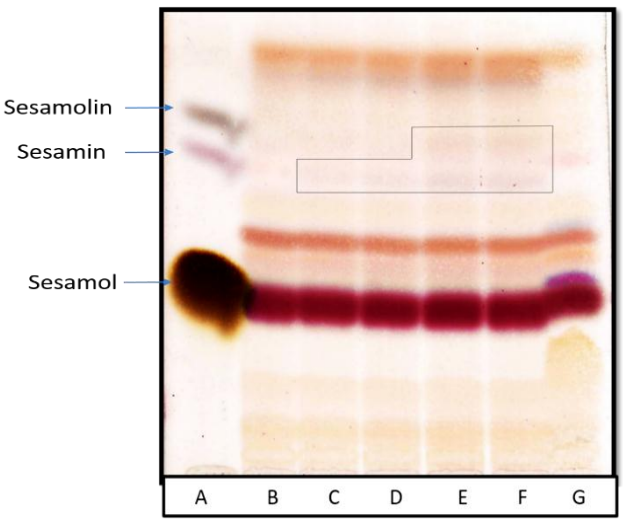


Fig.4.49 TLC for determination of detection level

A) Mix standard (sesamin, sesamol, sesamolin), B) 1% adulterated, C) 2% adulterated, D) 3% adulterated, E) 4% adulterated, F) 5% adulterated, G) Pure ghee

4.4.1.5 Profiling of sesamin and sesamolin of adulterated ghee samples on TLC during storage

4.4.1.5.1 Stability of sesamin and sesamolin in adulterated ghee samples (with and without BHA) during storage (37°C /8 months)

Adulterated ghee samples were prepared using vanaspati. Vanaspati was added to mixed ghee (cow ghee: buffalo ghee in the ratio of 1:1) individually at the rate of 1, 5, 10, 20 and 30% levels. Samples were stored in two different type of packaging materials i.e. sample bottles and multilayer pouches. The adulterated ghee samples were stored at 37ºC for 8 months and samples were subjected to TLC after an interval of every 2 month. Unsaponifiable matter was extracted from the samples for the profiling of sesamin and sesamolin and applied on the TLC plates. It can be inferred from Fig 4.50 that sesamin and sesamolin were initially detected at minimum level of 5% adulteration from adulterated ghee samples with and without BHA. It is evident from Fig 4.50 to 4.52 that duration of storage of adulterated ghee samples did not show any adverse effects on profiling of sesamin and sesamolin in TLC.

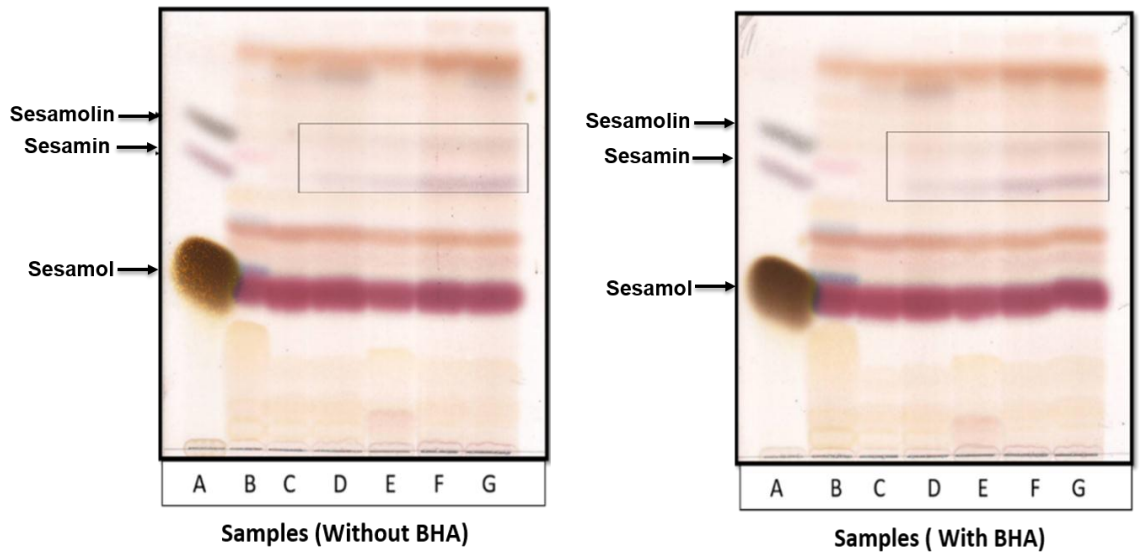


Fig.4.50 Profiling of sesamin and sesamolin in adulterated ghee samples on 4th month of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

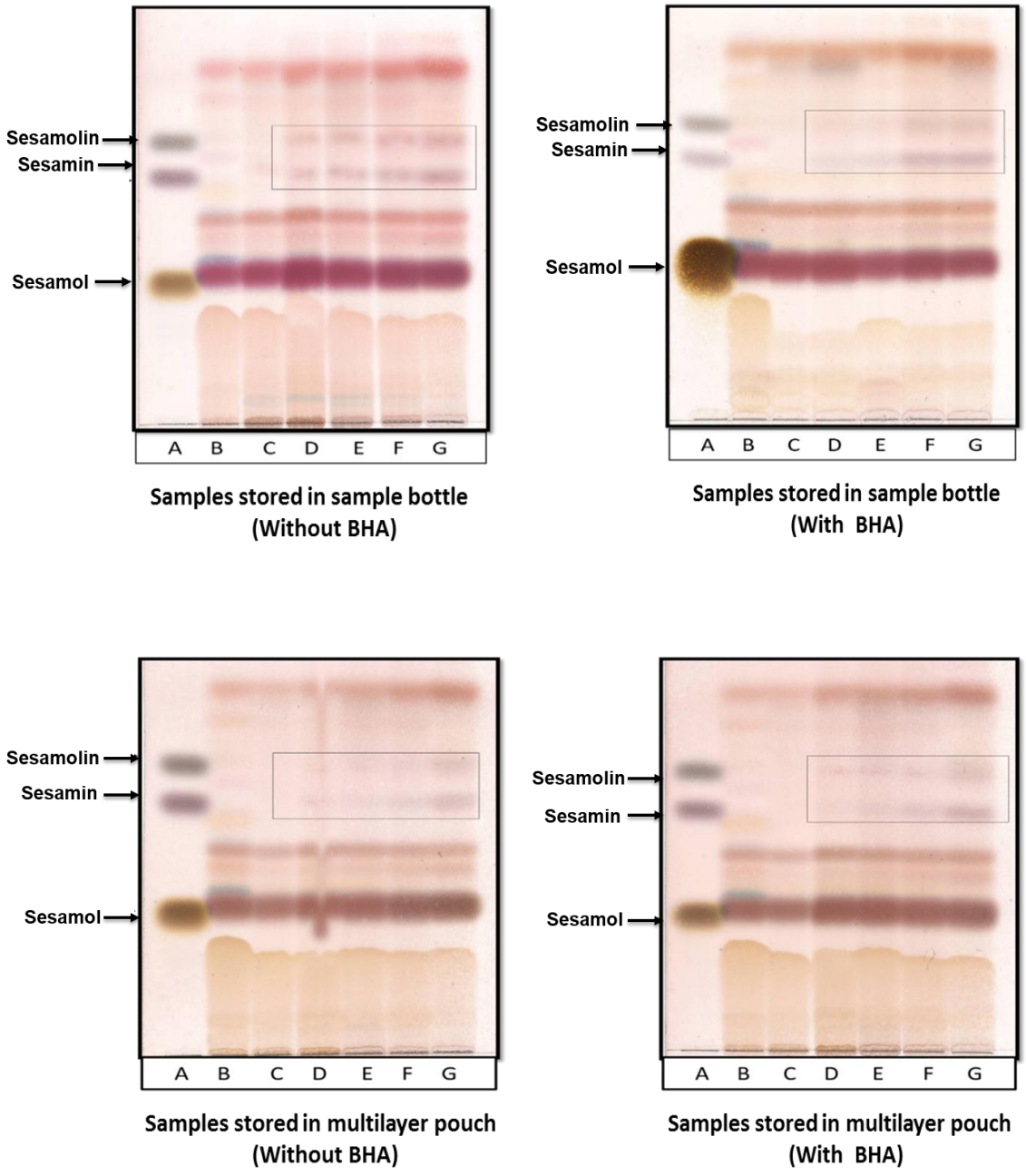


Fig.4.51 Profiling of sesamin and sesamolin in adulterated ghee samples on 6th month of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

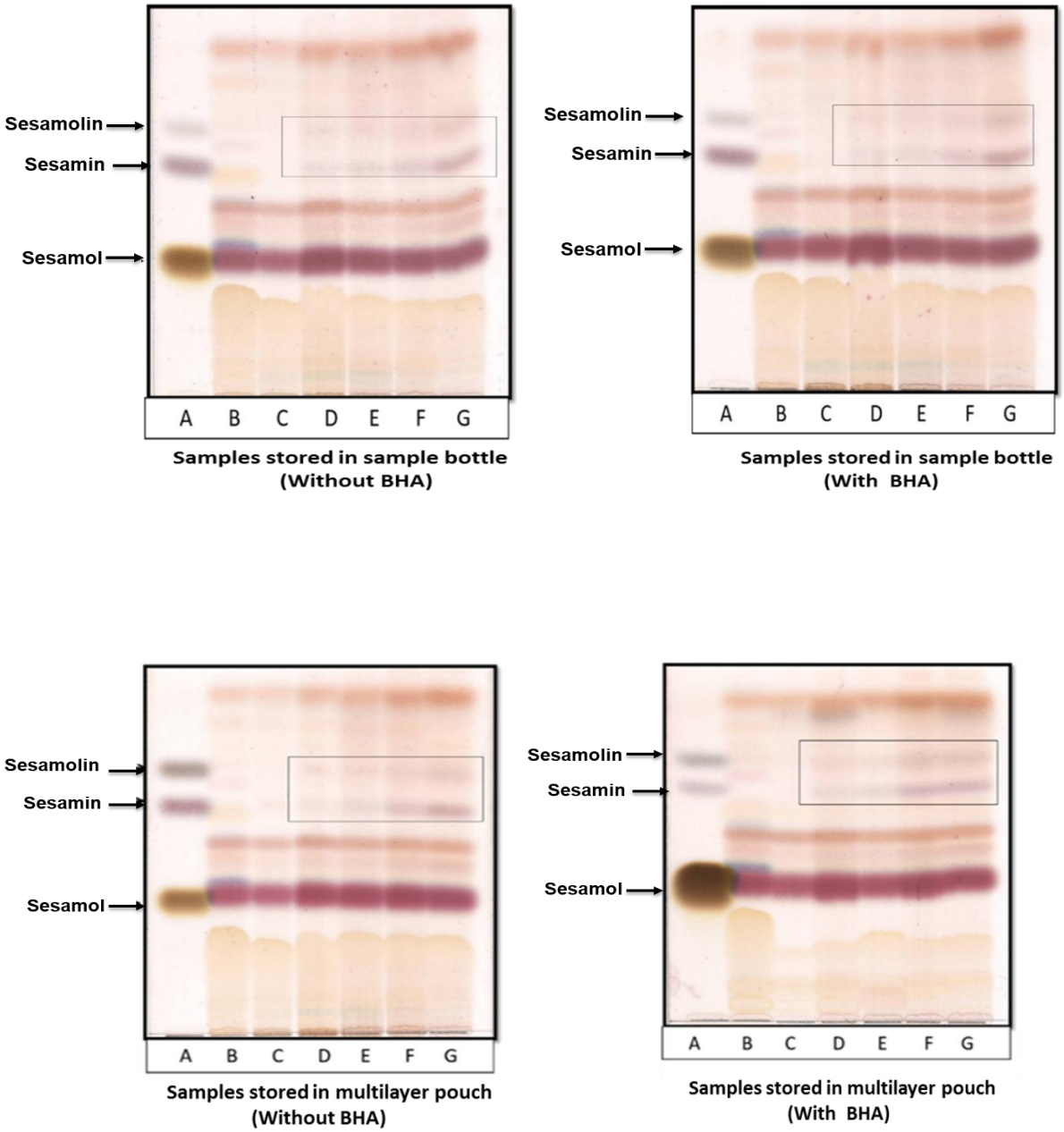


Fig.4.52 Profiling of sesamin and sesamolin in adulterated ghee samples on 8th month of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

4.4.1.5.2 Stability of sesamin and sesamolin in adulterated ghee samples (with and without BHA) during storage (80°C /10 days) (accelerated storage conditions)

Adulterated ghee samples in glass bottles were subjected to accelerated storage conditions (80ºC for 10 days). Stability of sasamin and sesamolin in adulterated ghee samples were evaluated after every 2 days. Unsaponifiable matter was extracted from the samples for the profiling of sesamin and sesamolin and applied on the TLC plates. It is evident from Fig 4.53 that sesamin and sesamolin were initially detected at minimum level of 5% adulteration in adulterated ghee samples with and without BHA. Unsaponifiable matter was extracted from the stored adulterated ghee samples and applied on TLC plates. It was clearly evident from Fig 4.53 to 4.58 that during storage of adulterated ghee samples (with and without addition of BHA), sesamin and sesamin were detected at minimum level of 5% adulteration of vanaspati in ghee. No adverse effect of duration of storage was observed on TLC profiling of sesamin and sesamolin of adulterated ghee samples throughout the storage at 80°C /10 days

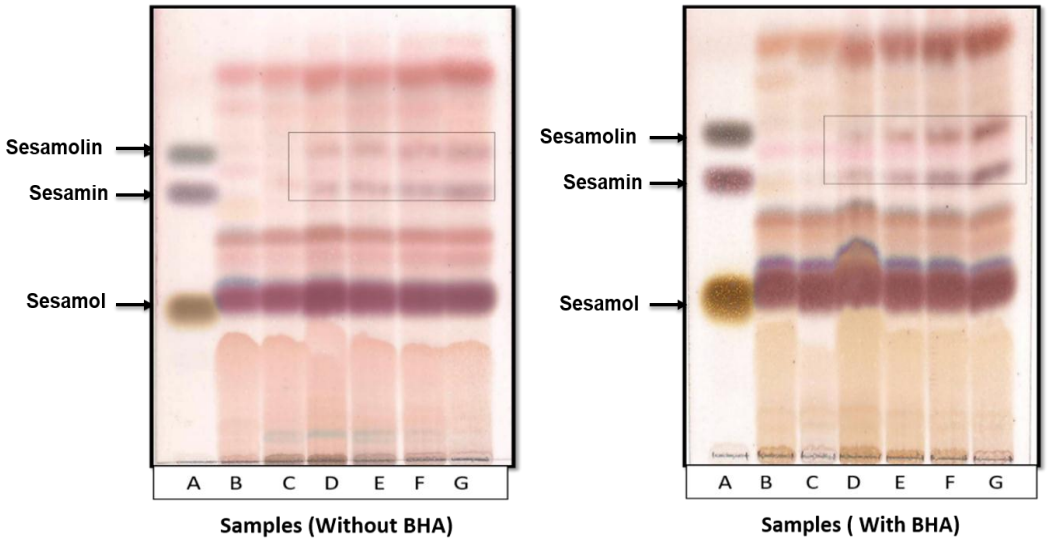


Fig.4.53 Profiling of sesamin and sesamin of adulterated ghee samples on 0 day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

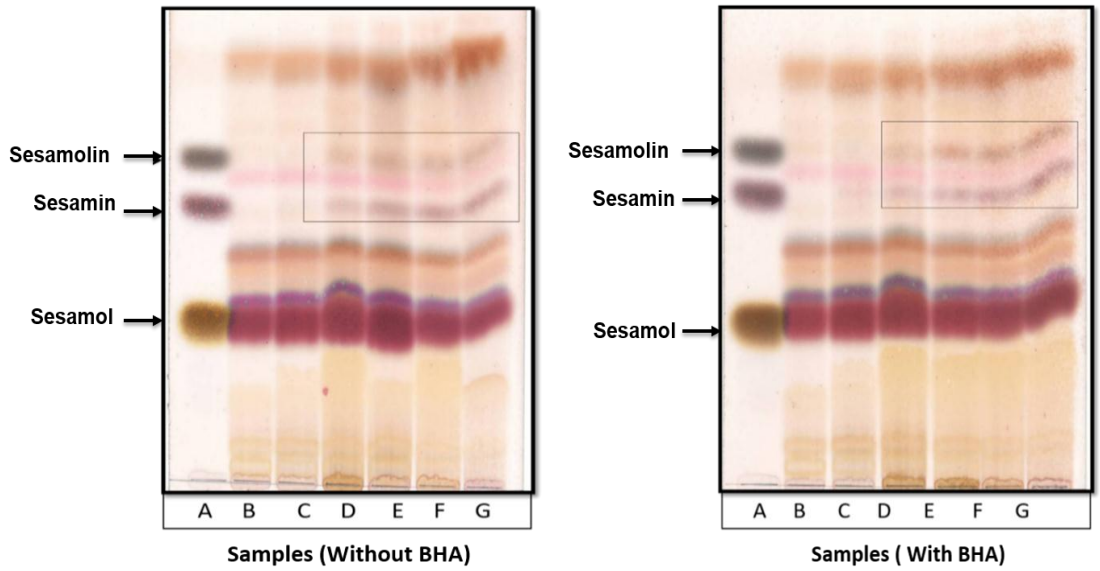


Fig.4.54 Profiling of sesamin and sesamin of adulterated ghee samples on 2nd day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

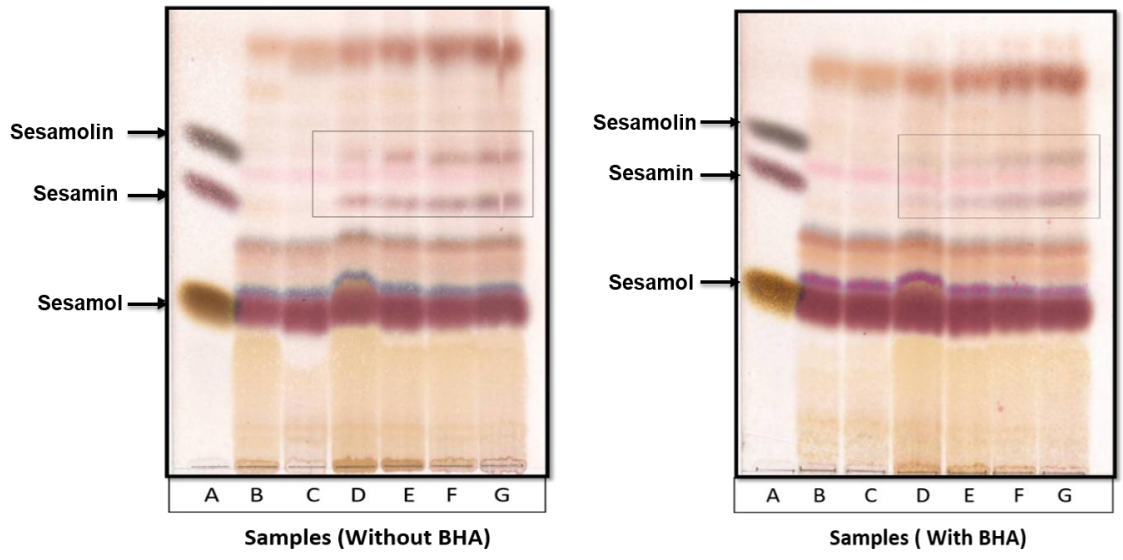


Fig.4.55 Profiling of sesamin and sesamin of adulterated ghee samples on 4th day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

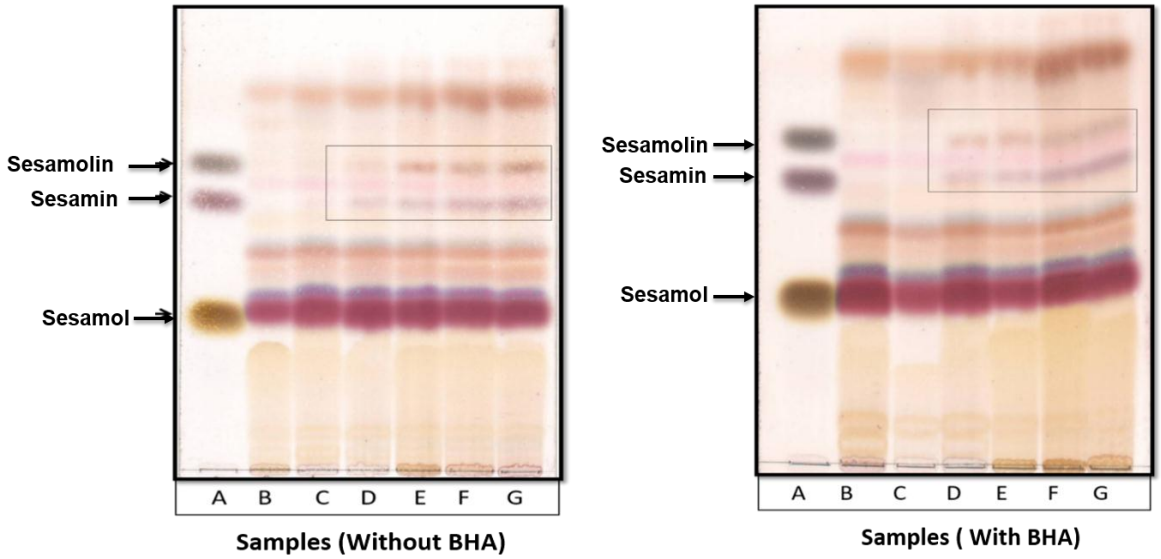


Fig.4.56 Profiling of sesamin and sesamin of adulterated ghee samples on 6th day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

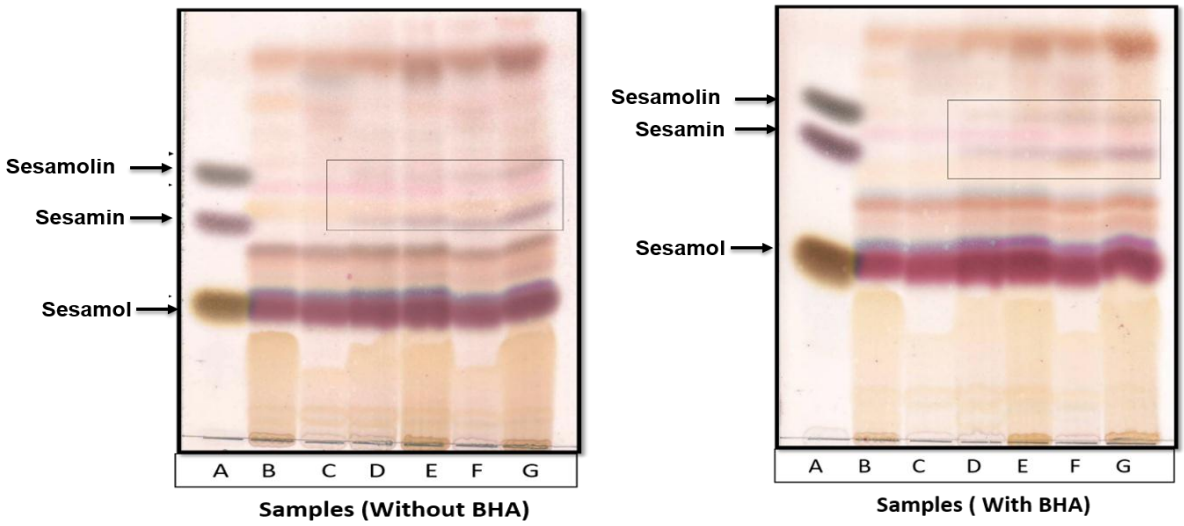


Fig.4.57 Profiling of sesamin and sesamin of adulterated ghee samples on 8th day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

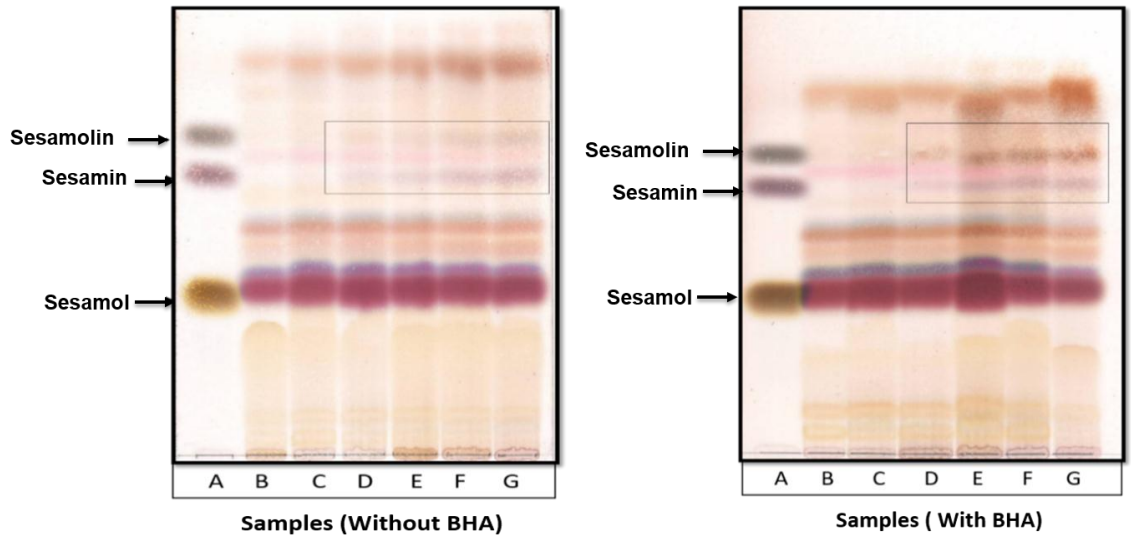


Fig.4.58 Profiling of sesamin and sesamin of adulterated ghee samples on 10th day of storage

A) Mix standard (sesamin, sesamol, sesamolin), B) Pure ghee, C) 1% adulterated, D) 5% adulterated, E) 10% adulterated, F) 20% adulterated, G) 30% adulterated

Results of TLC clearly demonstrated that it can be used for further confirmation of vanaspati in ghee. TLC profiling of sesamin and sesamolin can be used as a foolproof test, in case there is any variation in the results of Baudouin test between two testing laboratories of FSSAI.

4.4.2 HPLC analysis of adulterated ghee samples stored at 80ºC for 10 days

Analysis of sesamin and sesamolin was based on the method described by Schwertner and Rios (2012). They carried out detection of sesamin and sesamolin with Photodiode Array Detector (λmax=290 nm) and Fluorescent Detector (Ex.290 and Em.320). Analysis of sesamin and sesamolin was carried out according to method of Schwertner and Rios (2012) in case of USM extracted from ghee adulterated with vanaspati. It was observed that PDA detector was unable to detect the presence of sesamin and sesamolin in ghee samples adulterated with 1% vanaspati, whereas, detection with Fluorescent Detector gave appropriate results for ghee samples adulterated with 1% vanaspati. Use of Fluorescent Detector resulted in higher detection limits of analyte, hence, detection with Fluorescent Detector was selected for further

analysis. The adulterated ghee samples stored at 80ºC for 10 days were subjected to HPLC analysis for the estimation of degradation in tracer compounds (Sesamin and Sesamolin) and results were correlated with Baudouin and Sesamin test results.

4.4.2.1 Method for isolation of sesamin and sesamolin

Sesamin and sesamolin from adulterated ghee sample were isolated according to method of IS: 3508 (1966). In this method adulterated ghee samples was saponified with 5% methanolic KOH and extracted using diethyl ether.

4.4.2.2 HPLC analysis

4.4.2.2.1 Separation of sesamin and sesamolin

HPLC analytical conditions as listed earlier under material and methods section 3.9.2 was used for the separation of sesamin and sesamolin.

Fig 4.59 and Fig 4.60 represents the chromatogram for sesamin and sesamolin, respectively. Two different 'ratios of mobile phase' or 'mobile phase combinations' were chosen for estimation of sesamin and sesamolin i.e. methanol: water ratios (70:30 and 80:20). Table 4.16 enlists the retention time (RT) of sesamin and sesamolin with different mobile phase combinations. Methanol: water ratio (70:30) was selected as the HPLC mobile phase because it effectively resolved sesamin and sesamolin than methanol: water (80:20). Our results were also correlated with method described by Schwertner and Rios (2012). They concluded that the mobile phase, methanol: water ratio 70:30 gave effective resolution of sesamin and sesamolin as compared to methanol: water ratio 80:20. Three different flow rates (0.6, 0.8 and 1.0 ml/min) were assayed and the retention time of sesamin and sesamolin are presented in table 4.16. Thus, mobile phase methanol: water ratio 70:30 with flow rate 0.8 ml/min gave the best resolution as compared to other combinations.

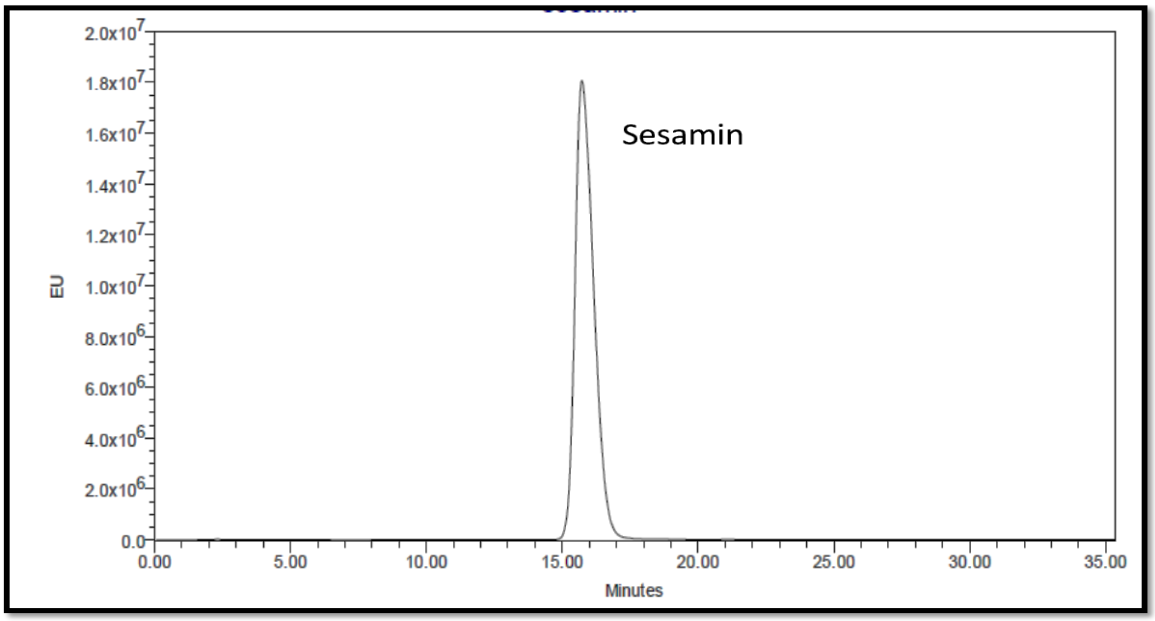


Fig. 4.59 HPLC Chromatogram of sesamin [15.19 min]

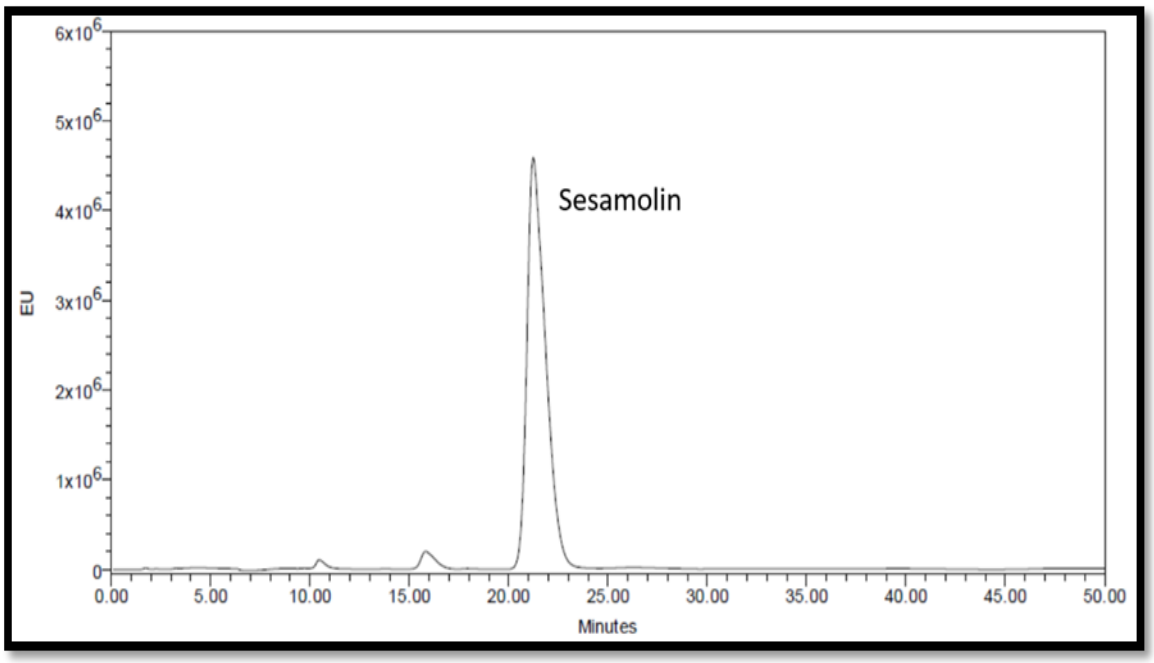


Fig. 4.60 HPLC Chromatogram of sesamolin [20.24 min]

Table 4.16 Retention time of sesamin and sesamolin with different mobile phase combinations

Sr. No.	Mobile phase combination ($CH_3OH:H_2O$)	Retention time (min)	
		Sesamin	Sesamolin
1	70:30	15.19	20.24
2	80:20	7.19	9.920

Table 4.17 Retention time of sesamin and sesamolin with different flow rate

Sr. No.	Flow rate (ml/min)	Retention time (min)	
		Sesamin	Sesamolin
1	0.6	21.06	21.28
2	0.8	15.19	20.24
3	1.0	13.26	17.95

Table 4.18 Retention times and areas of sesamin and sesamolin standards

Sr. No.	Compound	Retention time (min)	Area (0.2 mg/ml)
1	Sesamin	15.19	8694569280362
2	Sesamolin	20.24	2975669726959

4.4.2.2.2 Calibration curve

Linearity of sesamin and sesamolin were obtained over a concentration range of 0.001-0.2 mg/ml. The regression equations and correlations coefficient obtained are presented in table 4.19. The correlation coefficient of the calibration curve were better than 0.99.

Table 4.19 Regression equation and correlation coefficient of sesamin and sesamolin

Sr. No.	Compound	Regression equation	R^2 Correlation Coefficient
1	Sesamin	y = 2E+12x - 2E+12	0.998538
2	Sesamolin	y = 6E+11x - 6E+11	0.991825

4.4.2.3 Quantification procedure of sesamin and sesamolin over HPLC

4.4.2.3.1 Sample size

Three different sample sizes i.e. 0.2, 1 and 5 g were used for the sample preparation for detection of sesamin and sesamolin. Peak area and peak height of sesamin and sesamolin were less in case of 0.2 g as well as in 1 g of sample and higher in case of 5 g sample size. However, 5 g sample size produced sufficient peak area and peak height for detection and quantification. Therefore, 5g sample size was finally selected for further detection of sesamin and sesamolin in adulterated ghee samples.

4.4.2.3.2 Recovery experiments

Recovery experiments were performed for sesamin and sesamolin in the adulterated ghee samples. Mean recovery of the target compound was investigated by adding known concentrations of sesamin and sesamolin standard to the adulterated ghee samples. It is evident from table 4.20 and 4.21 that recovery was more than 97% in all the samples, which clearly indicated that extraction procedure was efficient for the isolation of sesamin and sesamolin.

The percent recovery of sesamin and sesamolin from adulterated ghee samples is reported in the table below

Table 4.20 Recovery (%) of sesamin from ghee samples adulterated with vanaspati

Sr. No.	Level of adulteration(% w/w)	% Recovery
1	1	98.81±0.27
2	5	98.45±0.17
3	10	97.58±0.13
4	20	98.24±0.03
5	30	98.43±0.06

Table 4.21 Recovery (%) of sesamolin from ghee samples adulterated with vanaspati

S. No.	Level of adulteration(% w/w)	% Recovery
1	1	98.62±0.27
2	5	98.74±0.31
3	10	98.92±0.03
4	20	98.93±0.03
5	30	98.44±0.16

4.4.2.4 Analysis of adulterated ghee samples during accelerated storage (80°C for 10 days)

The adulterated ghee samples were stored at 80°C for 10 days to investigate the changes occurring in the concentration of sesamin and sesamolin during high temperature of storage. Adulterated ghee samples were analysed at

the time interval of 2 days during storage period of 10 days. Sesamin and sesamolin isolated from the adulterated ghee samples were analysed over HPLC. A blank experiment was also run simultaneously with pure mixed ghee. Figures (4.61 to 4.70) represent the HPLC chromatograms of pure ghee and adulterated ghee samples under investigation during definite storage intervals.

4.4.2.4.1 Stability of sesamin and sesamolin in ghee samples adulterated with 1% vanaspati during storage (80°C for 10 days)

The stability of sesamin and sesamolin in ghee samples adulterated with 1 % vanaspati during storage were examined over HPLC. It is evident from the Table 4.22 and 4.23 that the sesamin and sesamolin levels significantly decreased ($P<0.05$) during storage at 80°C. Tables 4.22 shows that sesamin content of adulterated ghee samples (without BHA) decreased significantly during a time interval of 2 days but adulterated ghee samples (with BHA) showed lower rate of sesamin degradation than the adulterated ghee samples (without BHA). The lower degradation rate of sesamin in case of adulterated ghee samples (with BHA) may be due to the presence of BHA, which prevented oxidation of sesamin during storage. Similar trend was observed in case of sesamolin. Table 4.23 shows that the degradation of sesamolin in adulterated ghee samples (without BHA) was more than adulterated ghee samples (with BHA). As discussed earlier it may be due to the presence of antioxidant, which prevented the degradation of sesamolin. Table 4.22 and 4.23 indicates that the degradation of sesamin was lower than the degradation of sesamolin. Hemalatha (2007) concluded that sesamin was more stable during heat treatment than sesamolin which supported our findings. It was observed from the Table 4.22 that the amount of sesamin decreased significantly ($P<0.05$) from 98.81±0.27 to 96.02±0.04% in adulterated ghee samples (without BHA) and 98.81±0.29 to 97.99±0.01% in adulterated ghee samples (with BHA) during storage (80ºC for 10 days). Table 4.23 revealed that the amount of sesamolin decreased significantly ($P<0.05$) from 98.62±0.27 to 91.17±0.16 % in adulterated ghee samples (without BHA) and 98.74±0.29 to 98.00±0.07 % in adulterated ghee samples (with BHA). It was observed that degradation of sesamolin was more than the degradation of sesamin. Our results correlated well with the observations of Wu (2007) who reported that heating at 200ºC for 20 min caused

a significant loss of sesamolin and sesamol, whereas sesamin was found relatively heat stable even heating above 200ºC temperature.

Fig.4.61 represents the HPLC chromatogram of sesamin and sesamolin extracted from 1% vanaspati adulterated ghee sample (without BHA). Significant decrease ($P<0.05$) in sesamin and sesamolin content was observed during storage. Fig.4.62 shows the HPLC chromatogram of USM extracted from ghee sample (with BHA) adulterated with 1% vanaspati, which clearly points to the stability of sesamin and sesamolin during the storage. Similar trend was observed in other adulterated ghee samples, both with and without BHA. However, degradation of sesamin and sesamolin did not affect the Baudouin and Sesamin test throughout the storage as discussed earlier (section 4.2)

Table 4.22 Stability of sesamin in ghee adulterated with 1% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 1% vanaspati		
Storage period (days)	**Amount remaining of sesamin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.81 ± 0.27^{aA}	98.81 ± 0.29^{aA}
2	97.23 ± 0.08^{bB}	98.83 ± 0.06^{aA}
4	97.37 ± 0.07^{bC}	98.50 ± 0.04^{aB}
6	97.00 ± 0.04^{Bd}	98.14 ± 0.02^{aB}
8	96.50 ± 0.17^{bE}	98.09 ± 0.46^{aC}
10	96.02 ± 0.04^{bF}	97.99 ± 0.01^{aC}

Data are presented as means±SEM (n=3)

[a-b]Means within rows with different lowercase superscript are significantly different ($P<0.05$) from each other

[A-F]Means within column with different uppercase superscript are significantly different ($P<0.05$) from each other

Table 4.23 Stability of sesamolin in ghee adulterated with 1% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 1% vanaspati		
Storage period (days)	**Amount remaining of sesamolin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.62 ± 0.27^{aA}	98.74 ± 0.29^{aA}
2	95.60 ± 0.07^{bB}	98.63 ± 0.03^{aA}
4	94.59 ± 0.11^{bC}	98.54 ± 0.03^{aA}
6	93.04 ± 0.04^{bD}	98.26 ± 0.02^{aB}
8	92.03 ± 0.03^{bE}	98.03 ± 0.01^{aB}
10	91.17 ± 0.16^{bF}	98.00 ± 0.07^{aB}

Data are presented as means±SEM (n=3)

[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other

[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

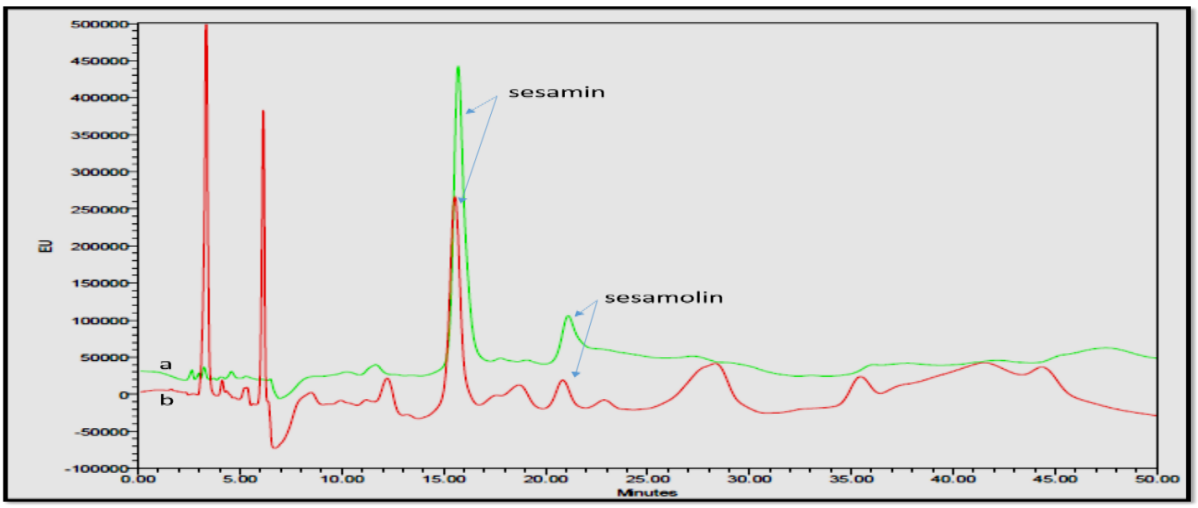


Fig. 4.61 HPLC Chromatogram of ghee (without BHA) adulterated 1% vanaspati

Where,

a= chromatogram of 0^{th} day storage

b= chromatogram of 10^{th} day storage

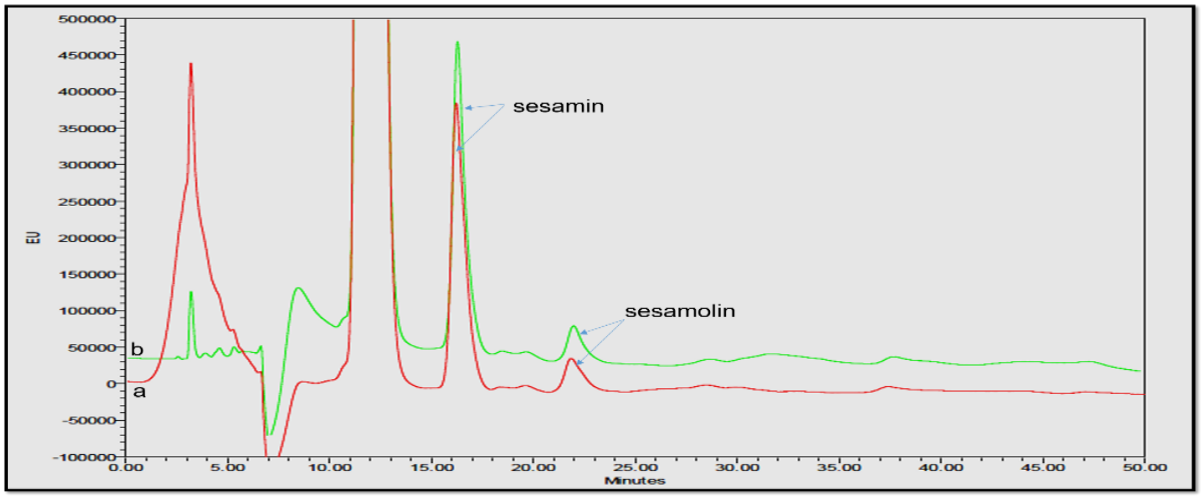


Fig. 4.62 HPLC Chromatogram of ghee (with BHA) adulterated 1% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

4.4.2.4.2 Stability of sesamin and sesamolin in ghee adulterated with 5% vanaspati samples during storage (80°C for 10 days)

Stability of sesamin and sesamolin in ghee samples adulterated with 5% vanaspati during storage at 80°C are represented in tables 4.24 and 4.25. It was observed that the stability of sesamin and sesamolin in ghee samples adulterated with 5% vanaspati significantly ($P<0.05$) decreased during storage at 80°C. Adulterated ghee samples (with BHA) showed more percentage retention of sesamin and sesamolin than adulterated ghee samples (without BHA). It is evident from table 4.25 that the amount of sesamin decreased significantly ($P<0.05$) from 98.45±0.17 to 93.32±0.22% in adulterated ghee samples (without BHA) and 98.75±0.21 to 97.69±0.10% in adulterated ghee samples (with BHA) during storage (80ºC for 10 days). Table 4.25 depicted that amount of sesamolin was significantly decreased from 98.74±0.31 to 88.99±0.44% in adulterated ghee samples (without BHA) and 99.05±0.08 to 96.00±0.01% in adulterated ghee samples (with BHA)

Table 4.24 Stability of sesamin in ghee adulterated with 5% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 5% vanaspati		
Storage period (days)	**Amount remaining of sesamin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.45±0.17^{aA}	98.75±0.21^{aA}
2	96.47±0.08^{bB}	98.71±0.01^{aA}
4	95.50±0.18^{bC}	98.51±0.02^{aA}
6	95.33±0.20^{bC}	98.28±0.03^{aB}
8	94.49±0.25^{bD}	98.06±0.01^{aB}
10	93.32±0.22^{bE}	97.69±0.10^{aC}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

Table 4.25 Stability of sesamolin in ghee adulterated with 5% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 5% vanaspati		
Storage period (days)	**Amount remaining of sesamolin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.74±0.31^{bA}	99.05±0.08^{aA}
2	94.47±0.07^{bB}	98.32±0.15^{aB}
4	93.50±0.18^{bC}	98.36±0.17^{aB}
6	91.33±0.19^{bD}	97.04±0.03^{aC}
8	90.49±0.25^{bE}	96.61±0.08^{aD}
10	88.99±0.44^{bF}	96.00±0.01^{aE}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

Fig.4.63 and Fig 4.64 represents the HPLC chromatograms of USM extracted from ghee sample (with and without BHA) adulterated with 5% vanaspati. Sesamin and sesamolin peaks of the fresh adulterated ghee samples were compared with the stored adulterated ghee samples in which chromatograms indicated the degradation of sesamin and sesamolin during storage in adulterated ghee samples (with and without BHA). It was observed that adulterated ghee samples (with BHA) had lower degradation of sesamin and sesamolin. Although degradation of sesamin and sesamolin did not affect the Baudouin and Sesamin test throughout the storage.

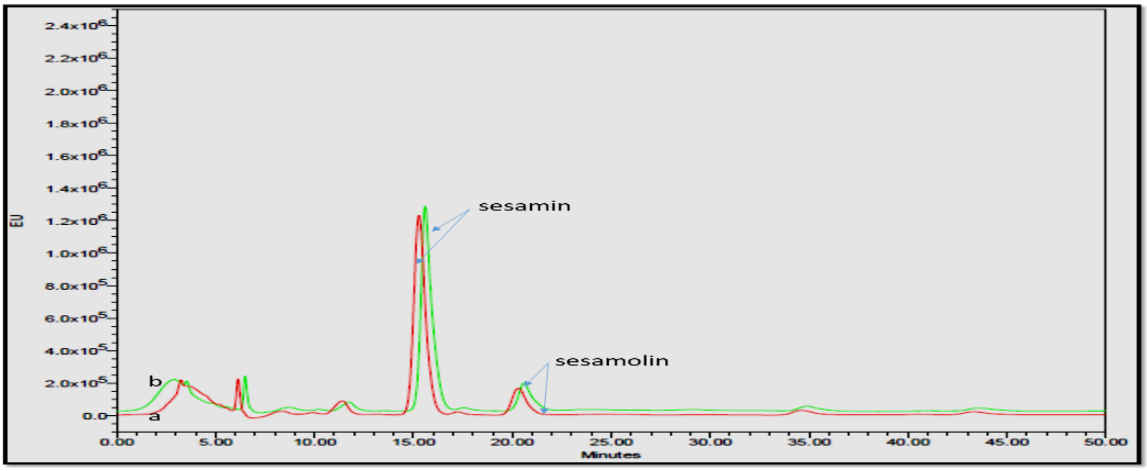


Fig. 4.63 HPLC Chromatogram of ghee (without BHA) adulterated 5% vanaspati

Where, a = chromatogram of 0 day storage
B = chromatogram of 10th day storage

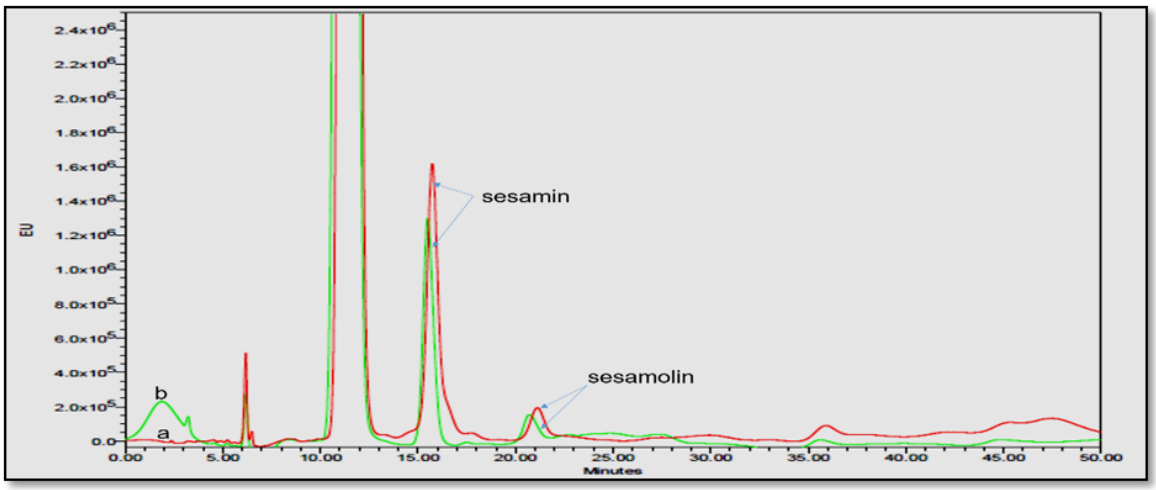


Fig. 4.64 HPLC Chromatogram of ghee (with BHA) adulterated 5% vanaspati

Where, a = chromatogram of 0 day storage
B = chromatogram of 10th day storage

4.4.2.4.3 Stability of sesamin and sesamolin in 10% adulterated ghee samples during storage at 80°C for 10 days

Tables 4.26 and 4.27 indicated that the sesamin and sesamolin content of the adulterated ghee samples (with and without BHA) decreased significantly ($P<0.05$) during storage at 80°C. It was observed from the Table 4.26 that the amount of sesamin decreased significantly ($P<0.05$) from 97.58±0.13 to 90.29±0.07 % in adulterated ghee samples (without BHA) and 97.69±0.24 to 96.11±0.06% in adulterated ghee samples (with BHA) during storage (80ºC for 10 days). It was interpreted from the Table 4.27 revealed that the amount of sesamolin decreased significantly ($P<0.05$) from 98.92±0.03 to 85.04±0.03% in adulterated ghee samples (without BHA) and 98.88±0.06 to 94.09±0.01 % in adulterated ghee samples (with BHA) which clearly indicated that the degradation of sesamolin was more in case of adulterated ghee sample (without BHA). It was observed that the degradation of sesamolin did not affect the result of Baudouin test but it was observed that degradation of sesamin during storage affected Sesamin test. On 10^{th} day of storage Sesamin test gave conflicting results for ghee samples adulterated with 10% vanaspati (without BHA) whereas no adverse effect of storage was observed in 10% adulterated ghee samples (with BHA). The results revealed that sesamolin degraded more than sesamin during the storage, although there was no adverse effect observed on Baudouin test, this could be due to of formation of sesamol which can result in positive Baudouin test (sesamol and sesamolin both are able to give red colour in Baudouin test) whereas, degradation of sesamin did not form any related compound which can give positive Sesamin test during the storage

Table 4.26 Stability of sesamin in ghee adulterated with 10% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 10% vanaspati		
Storage period (days)	**Amount remaining of sesamin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	97.58 ± 0.13^{aA}	97.69 ± 0.24^{aA}
2	94.70 ± 0.16^{bB}	97.16 ± 0.03^{aB}
4	93.49 ± 0.15^{bC}	96.90 ± 0.07^{aC}
6	93.05 ± 0.06^{bD}	96.61 ± 0.03^{aD}
8	91.68 ± 0.06^{bE}	96.43 ± 0.05^{aE}
10	90.29 ± 0.07^{bF}	96.11 ± 0.06^{aF}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

Table 4.27 Stability of sesamolin in ghee adulterated with 10% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 10% vanaspati		
Storage period (days)	**Amount remaining of sesamolin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.92 ± 0.03^{aA}	98.88 ± 0.06^{aA}
2	92.13 ± 0.02^{bB}	97.16 ± 0.09^{aB}
4	90.09 ± 0.05^{bC}	96.04 ± 0.04^{aC}
6	88.19 ± 0.07^{bD}	95.79 ± 0.10^{aD}
8	87.65 ± 0.05^{bE}	95.13 ± 0.11^{aE}
10	85.04 ± 0.03^{bF}	94.09 ± 0.01^{aF}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

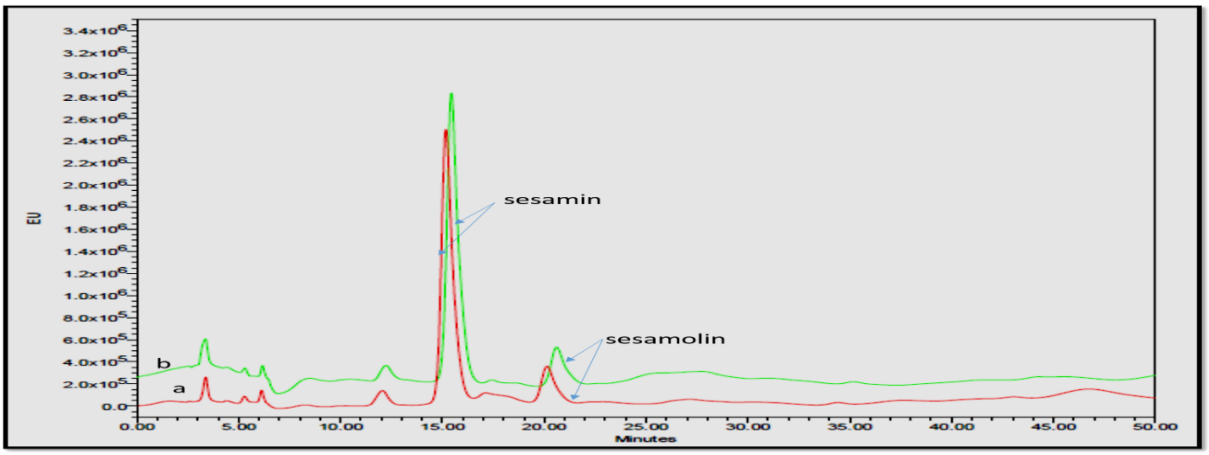


Fig. 4.65 HPLC Chromatogram of ghee (without BHA) adulterated 10% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

Fig.4.65 and 4.66 depicted the HPLC chromatograms of USM extracted from ghee samples (with and without BHA) adulterated with 10% vanaspati. It was evident from the chromatograms that sesamin and sesamolin content decreased during storage.

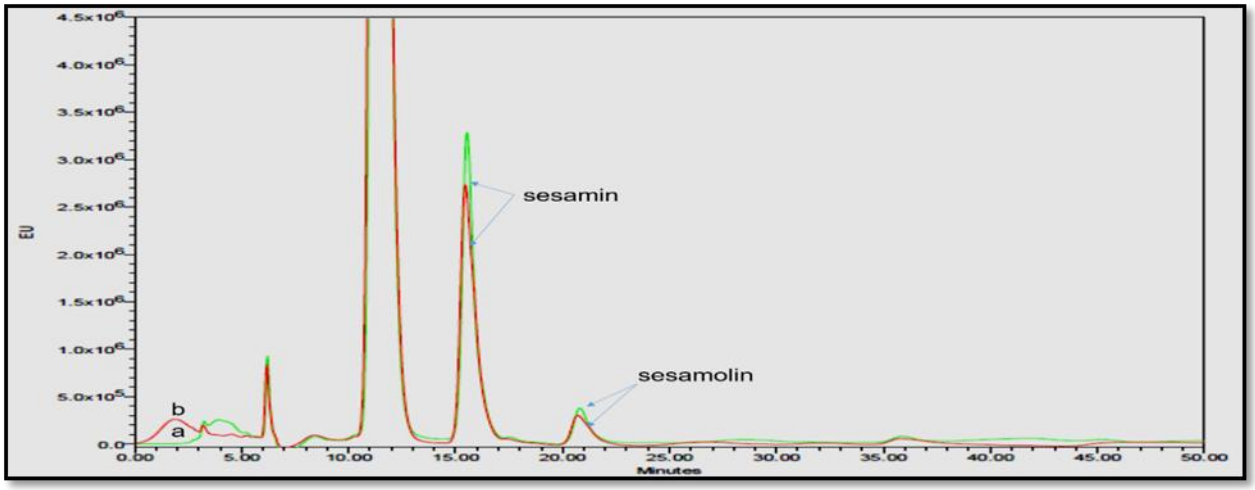


Fig. 4.66 HPLC Chromatogram of ghee (with BHA) adulterated 10% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

4.4.2.4.4 Stability of sesamin and sesamolin in ghee adulterated with 20% vanaspati during storage at 80°C for 10 days

The content of sesamin and sesamolin in 20% adulterated ghee samples during storage is shown in Tables 4.28 and 4.29. It was observed from the Table 4.28 that the amount of sesamin decreased significantly (P<0.05) from 98.24±0.03 to 86.80±0.11% in adulterated ghee samples (without BHA) and 98.83±0.08 to 93.01±0.02% in adulterated ghee samples (with BHA) during storage (80ºC for 10 days). Table 4.29 clearly showed that amount of sesamolin decreased significantly (P<0.05) from 98.93±0.03 to 80.12±0.05% in adulterated ghee samples (without BHA) and 98.90±0.05 to 90.52±0.01% in adulterated ghee samples (with BHA) which indicated that the degradation of sesamolin was more in case of adulterated ghee sample (without BHA). It was observed from Table 4.28 and 4.29 that the amount of sesamin and sesamolin significantly (P<0.05) decreased during storage. The HPLC chromatograms (Fig 4.67 and 4.68) also indicated that the content of sesamin and sesamolin decreased. Lower decrease in the amount of sesamin as well as sesamolin was observed in adulterated ghee samples (with BHA) as compared to the adulterated ghee samples (without BHA). Decrease in the amount of sesamin and sesamolin did not affect the Sesamin and Baudouin test.

Table 4.28 Stability of sesamin in ghee adulterated with 20% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 20% vanaspati		
Storage period (days)	**Amount remaining of sesamin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.24 ± 0.03^{bA}	98.83 ± 0.08^{aA}
2	94.36 ± 0.16^{bB}	98.01 ± 0.02^{aB}
4	92.14 ± 0.10^{bC}	97.12 ± 0.01^{aC}
6	90.42 ± 0.15^{bD}	96.11 ± 0.03^{aD}
8	88.80 ± 0.10^{bE}	95.35 ± 0.01^{aE}
10	86.80 ± 0.11^{bF}	93.01 ± 0.02^{aF}

Data are presented as means±SEM (n=3)

$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other

$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

Table 4.29 Stability of sesamolin in ghee adulterated with 20% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 20% vanaspati		
Storage period (days)	**Amount remaining of sesamolin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.93±0.03^{aA}	98.90±0.05^{aA}
2	89.10±0.44^{bB}	95.72±0.03^{aB}
4	85.60±0.31^{bC}	94.02±0.03^{aC}
6	82.52±0.56^{bD}	92.14±0.02^{aD}
8	81.94±0.13^{bD}	91.13±0.01^{aE}
10	80.12±0.05^{bE}	90.52±0.01^{aF}

Data are presented as means±SEM (n=3)
$^{a-b}$Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
$^{A-F}$Means within column with different uppercase superscript are significantly different (P<0.05) from each other

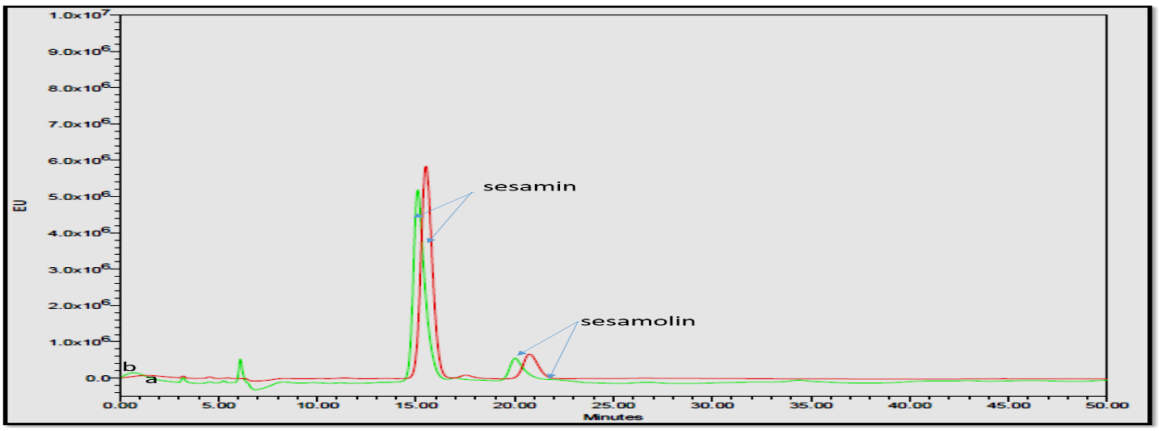


Fig. 4.67 HPLC Chromatogram of ghee (without BHA) adulterated 20% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

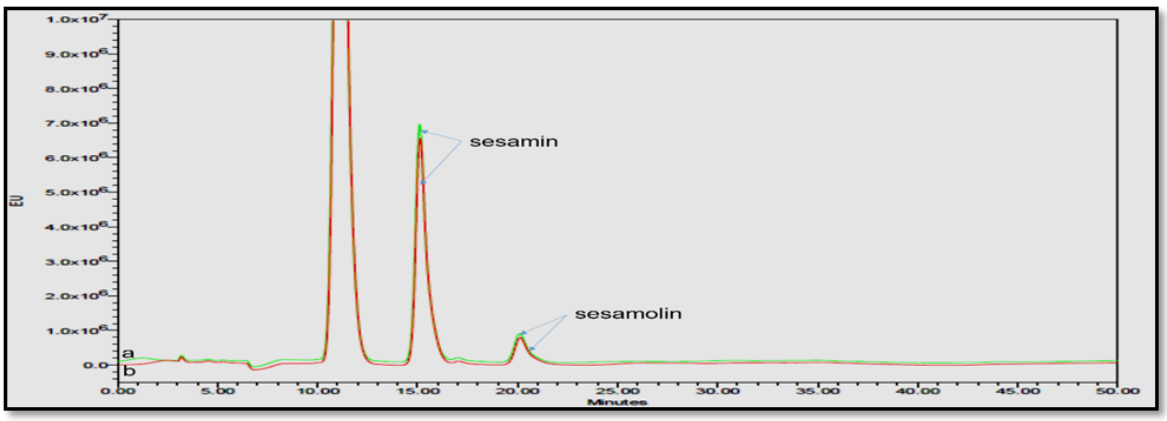


Fig. 4.68 HPLC Chromatogram of ghee (with BHA) adulterated 20% vanaspati

Where,

a= chromatogram of 0 day storage
b= chromatogram of 10th day storage

4.4.2.4.4 Stability of sesamin and sesamolin in ghee adulterated with 30% vanaspati during storage at 80°C for 10 days

The content of sesamin and sesamolin in 30% adulterated ghee samples during storage is shown in Tables 4.30 and 4.31. It was observed from the tables that the sesamin and sesamolin levels significantly ($P<0.05$) decreased during storage at 80°C. It was observed from the Table 4.30 that the amount of sesamin decreased significantly ($P<0.05$) from 98.43±0.06 to 84.52±0.05% in adulterated ghee samples (without BHA) and 98.47±0.12 to 91.11±0.07% in adulterated ghee samples (with BHA) during storage (80ºC for 10 days). Table 4.31 clearly showed that the amount of sesamolin decreased significantly ($P<0.05$) from 98.44±0.16 to 77.49±0.10% in adulterated ghee samples (without BHA) and 98.43±0.07 to 81.52±0.05% in adulterated ghee samples (with BHA) which indicated that the degradation of sesamolin was more in case of adulterated ghee sample (without BHA).

Adulterated ghee samples (with BHA) showed more stability of sesamin and sesamolin than adulterated ghee samples (without BHA). It was evident from table 4.30 and 4.31 that sesamin and sesamolin content after storage was more in adulterated ghee samples (with BHA) than adulterated ghee samples (without BHA).

Table 4.30 Stability of sesamin in ghee adulterated with 30% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 30% vanaspati		
Storage period (days)	**Amount remaining of sesamin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.43 ± 0.06^{aA}	98.47 ± 0.12^{aA}
2	93.19 ± 0.09^{bB}	95.21 ± 0.11^{aB}
4	91.56 ± 0.04^{bC}	94.43 ± 0.12^{aC}
6	88.72 ± 0.16^{bD}	93.00 ± 0.06^{aD}
8	87.13 ± 0.05^{bE}	92.04 ± 0.04^{aE}
10	84.52 ± 0.05^{bF}	91.11 ± 0.07^{aF}

Data are presented as means±SEM (n=3)
[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

Table 4.31 Stability of sesamolin in ghee adulterated with 30% vanaspati during storage at 80°C for 10 days

Ghee adulterated with 30% vanaspati		
Storage period (days)	**Amount remaining of sesamolin (%)**	
	Ghee (without BHA)	**Ghee (with BHA)**
0	98.44 ± 0.16^{aA}	98.43 ± 0.07^{aA}
2	87.81 ± 0.03^{bB}	91.19 ± 0.09^{aB}
4	85.22 ± 0.02^{bC}	88.56 ± 0.04^{aC}
6	83.72 ± 0.01^{bD}	85.72 ± 0.16^{aD}
8	82.01 ± 0.02^{bE}	84.13 ± 0.05^{aE}
10	77.49 ± 0.10^{bF}	81.52 ± 0.05^{aF}

Data are presented as means±SEM (n=3)
[a-b]Means within rows with different lowercase superscript are significantly different (P<0.05) from each other
[A-F]Means within column with different uppercase superscript are significantly different (P<0.05) from each other

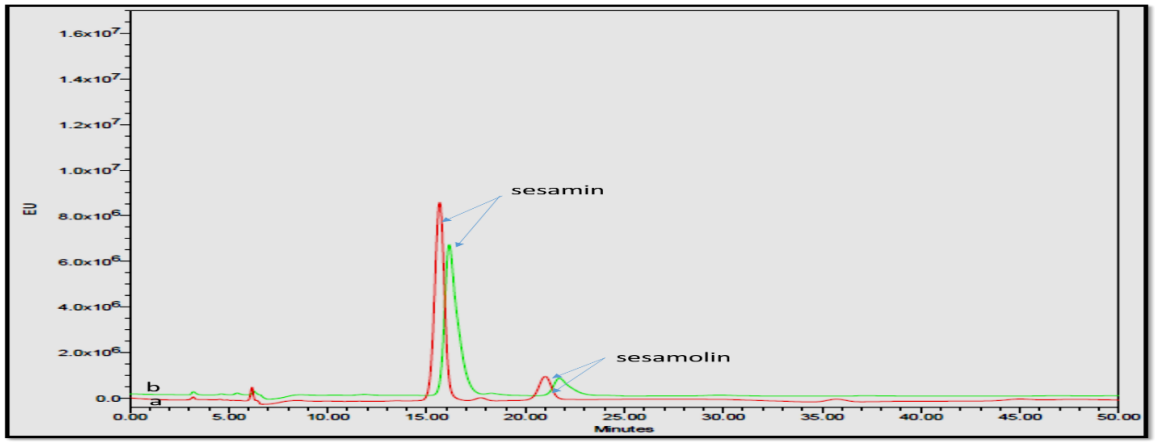


Fig. 4.69 HPLC Chromatogram of ghee (without BHA) adulterated 30% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

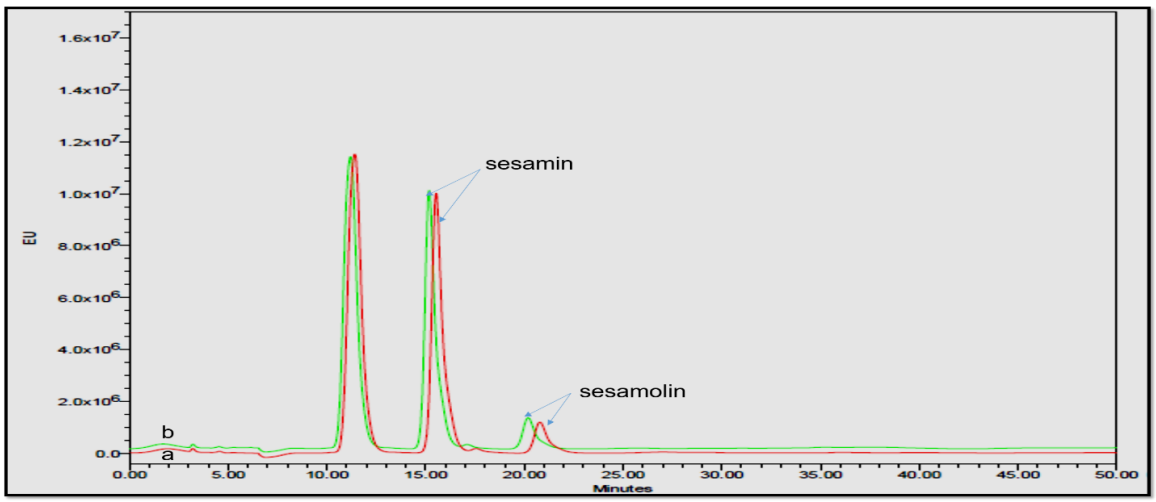


Fig. 4.70 HPLC Chromatogram of ghee (with BHA) adulterated 30% vanaspati

Where,

a= chromatogram of 0 day storage

b= chromatogram of 10th day storage

Fig.4.69 and 4.70 represents the HPLC chromatograms of USM extracted from 30% adulterated ghee samples (with and without BHA). It was evident from the chromatograms that sesamin and sesamolin content decreased during

storage. Sesamin and sesamolin significantly reduced ($P<0.05$) after storage at 80°C for 10 days in adulterated ghee samples (with and without BHA) however, the effect of this decrease on the Baudouin test was negligible which is earlier described in section 4.2

Initially at the beginning of storage, the Sesamin test gave positive results in ghee samples adulterated with 10, 20 and 30% vanaspati. However, after storage the positive results were obtained in ghee samples adulterated with 20 and 30% vanaspati only, whereas doubtful/conflicting results were obtained in 10% ghee samples adulterated with 10% vanaspati. The Baudouin test gave positive results result in ghee samples adulterated with 10, 20 and 30% vanaspati throughout the storage at 80ºC for 10 days.

CHAPTER –5

Summary and Conclusions

SUMMARY AND CONCLUSIONS

Ghee adulterated with vanaspati containing sesame oil can be easily detected using Baudouin test, where the appearance of red coloured complex indicates a positive test. A colour based sesamin test for the detection of sesame oil in vegetable oil has been approved by Codex (1999), however this test was not performed in case of ghee. Baudouin and Sesamin test are based on the presence of the sesame lignans i.e. sesamolin and sesamin, respectively. These sesame lignans tends to degrade with time which may cause the false negative results which can mislead and encourage adulteration and ultimately lead to acquittal of fraudulent traders. No work has been reported in literature for the evaluation of Baudouin and Sesamin test on the ghee samples during storage, as well as the effect of packaging materials on the degradation of sesamin and sesamolin. Hence, the present study was carried out to examine the effect of different storage conditions (37ºC for 8 months and 80ºC for 10 days), different packaging materials (i.e. sample bottles and multilayer pouches), presence of BHA on degradation of tracer compounds (i.e. sesamin and sesamolin) in adulterated samples and the impact of these factors on Baudouin and Sesamin test.

The study was carried out for:

- Profiling of tracer components (Sesamin and Sesamolin) using chromatographic procedures and correlation with Baudouin and Sesamin test
- To examine the efficacy of Baudouin and Sesamin test in vanaspati adulterated ghee with and without addition of BHA during storage

This chapter deals with the summary of the major findings of the present work.

1. Sesamin test was optimised with regard to the concentration of furfural solution used to detect the adulteration of vanaspati in ghee. Selection of the concentration of furfural solution was based on the development of clear bluish green colour indicating positive test results.
2. On the initial day of storage, Sesamin test showed positive results for ghee samples adulterated with vanaspati at higher concentrations (i.e. 10,

20 and 30%), whereas negative results were observed at lower concentrations (1 and 5%). Adulterated ghee samples (with and without BHA) gave positive Sesamin test results throughout the storage at 37ºC for 8 months. No adverse effect on Sesamin test result was observed for ghee adulterated with vanaspati, stored in different packaging materials (sample bottles and multilayer pouches) during storage at 37ºC for 8 months.

3. On the initial day of storage, Baudouin test showed positive results for ghee samples adulterated with vanaspati at higher concentrations (i.e. 10, 20 and 30%), whereas negative results were observed at lower concentrations (1 and 5%). Adulterated ghee samples with and without BHA gave positive Baudouin test results throughout the storage period (37ºC for 8 months), stored in different packaging materials (sample bottles and multilayer pouches).
4. Significant difference ($P<0.05$) was observed in peroxide value of pure ghee and adulterated ghee samples during storage (37ºC for 8 months). Increase in peroxide value was observed upon increasing the concentration of vanaspati in pure ghee (i.e. 1 to 30%). Maximum peroxide values were observed in samples stored in multilayer pouches (without BHA). Significant difference ($P<0.05$) was observed in peroxide value of samples with and without BHA. It was observed that ghee (without BHA) showed significantly higher ($P<0.05$) peroxide value during storage (37ºC for 8 months) than ghee (with BHA) due to the absence of antioxidant.
5. Profiling of sesamin and sesamolin was carried out with normal phase TLC, which gave an excellent separation of sesamin and sesamolin from the unsaponifiable matter of adulterated ghee. It was possible to detect the adulteration of vanaspati in ghee on the basis of sesamin and sesamolin as tracer components. Sesamin and sesamolin were detected on TLC @ 5, 10, 20 and 30% adulteration in ghee throughout the storage period (37ºC / 8 months and 80ºC / 10 days).
6. Ghee samples adulterated with vanaspati were stored under accelerated storage conditions (80ºC for 10 days) to examine the changes in Baudouin test. No adverse effect was observed on Baudouin test

throughout the storage period. Positive results at 10% level of adulteration and also at higher concentrations (20 and 30%) were obtained, whereas negative results were obtained at lower concentrations (1 and 5%) throughout the storage period (80ºC for 10 days).

7. Ghee samples adulterated with vanaspati were subjected to Sesamin test on the initial day of accelerated storage (80ºC for 10 days). Sesamin test gave positive results at 10% level of adulteration and also at higher concentrations (20 and 30%), whereas negative results were obtained at lower concentrations (1 and 5%). On the 10^{th} day of storage at 80ºC, Sesamin test gave conflicting results for 10% adulterated sample (without BHA), whereas for 10% adulterated sample (with BHA) results were not affected.
8. It was observed that ghee (without BHA) showed significantly higher ($P<0.05$) peroxide value than ghee (with BHA) due to the absence of antioxidant during storage (80ºC for 10days).
9. HPLC analysis of sesamin and sesamolin was carried out to examine the changes occurring in the concentration of sesamin and sesamolin during storage. Detection of sesamin and sesamolin was possible even at lower level of adulteration i.e. ghee adulterated with 1% vanaspati.
10. HPLC analysis of sesamin and sesamolin showed degradation of both the compounds during storage at 80ºC. A significant decrease ($P<0.05$) was observed in the content of sesamin and sesamolin in adulterated ghee samples between 0 day and 10^{th} day of storage at 80ºC. The adulterated ghee sample with BHA showed a significantly higher ($P<0.05$) content of sesamolin in comparison to the adulterated sample without BHA. A pronounced difference was observed in the degradation of sesamolin and sesamin, which led us to the conclusion that sesamin was relatively more stable than sesamolin during storage at 80ºC.
11. HPLC analysis of sesamin and sesamolin showed that minimum degradation of sesamin and sesamolin occurred in samples with BHA in comparison to samples without BHA. It was evident from the results that BHA exerted antioxidant effect i.e. prevented degradation of the tracer compounds (i.e. sesamin and sesamolin).

CONCLUSIONS

- The results of the present investigation showed that Baudouin test can be used for stored ghee sample in the detection of adulteration with vanaspati (containing sesame oil). It can also be interpreted that Sesamin test can be used to detect the adulteration of ghee with vanaspati (containing sesame oil) during storage. No effect of packaging material was observed on the Sesamin and Baudouin test, which showed the efficacy of Sesamin and Baudouin test to detect the adulteration of vanaspati in ghee during storage.
- It was observed that colour produced in Sesamin test was more intense than colour produced in Baudouin during the detection of ghee samples adulterated with vanaspati. It can therefore be suggested that Sesamin test can be used for the detection of vanaspati in ghee, as it gave intense colour for positive results which can be very helpful for the clear identification of adulterated ghee samples.
- It was also observed that sesamin and sesamolin were detected on TLC @ 5, 10, 20 and 30% vanaspati adulteration in ghee throughout the storage period (37ºC / 8 months and 80ºC / 10 days) which showed that detection of adulteration of vanaspati in ghee on the basis of profiling of sesamin and sesamolin was possible during the storage.
- Sesamin and sesamolin were detected using HPLC in ghee samples adulterated with vanaspati @ 1, 5, 10, 20 and 30% adulteration in ghee throughout the storage period (80ºC / 10 days). Results of HPLC analysis showed that the detection of ghee samples adulterated with vanaspati is possible during storage.
- Finally, it can be concluded that Baudouin and Sesamin test work efficiently to detect the presence of vanaspati in ghee during storage. The other detection methods (i.e. TLC and HPLC) used in the present study also can be used to detect the presence of vanaspati in ghee during storage and these chromatographic methods have relatively lower detection limits to detect the presence of vanaspati in ghee than Baudouin and Sesamin test.

Bibliography

BIBLIOGRAPHY

Amarowicz, R., Shahidi, F., and Pegg, R. B. (2001). Application of semipreparative RP-18 HPLC for the purification of sesamin and sesamolin. *Journal of Food Lipids*, 8(2), 85-94.

Adegbola, A. R., Dare, E. O., and Lasisi, A. A. (2008). Spray reagents for the visualization and detection of sesame oil unsaponifiables on thin-layer Chromatograms. *Chromatographia*, 68(1-2), 151-153.

Anon. (2014). http://timesofindia.indiatimes.com/city/coimbatore/50-of-ghee-adulterated-say-food-safety-officers/articleshow/45486643.cms, accessed on 26 August, 2015

Anon. (2015a). http://timesofindia.indiatimes.com/city/vijayawada/Ghee-maker-sealed-on-charge-of-adulteration/articleshow/47630240.cms, accessed on 6 January, 2016.

Anon. (2015b). http://timesofindia.indiatimes.com/city/vijayawada/Vijayawada-Ghee-adulteration-found/articleshow/49901424.cms, accessed on 3 March, 2016.

Arakawa, H., Nakabayashi, S., Ohno, K. I., and Maeda, M. (2012). New fluorimetric assay of horseradish peroxidase using sesamol as substrate and its application to EIA. *Journal of Pharmaceutical Analysis*, 2(2), 156-159.

Budowski, P., and Markley, K. S. (1951). The chemical and physiological properties of sesame Oil. *Chemical reviews*, 48(1), 125-151.

Buntinx, M., Willems, G., Knockaert, G., Adons, D., Yperman, J., Carleer, R., & Peeters, R. (2014). Evaluation of the thickness and oxygen transmission rate before and after thermoforming mono-and multi-layer sheets into trays with variable depth. *Polymers*, *6*(12), 3019-3043.

Bhatnagar, A. S., Hemavathy, J., and Krishna, A. G. (2015). Development of a rapid method for determination of lignans content in sesame oil. *Journal of Food Science and Technology*, 52(1), 521-527.

Codex (1999). Codex Alimentarius: fats, oils and related products; Standards for fats and oils from vegetable sources (CODEX-STAN 210-1999). Joint FAO/WHO food standard programme, Codex Alimentarius Commission, Rome, Italy.

Dar, A. A., Verma, N. K., and Arumugam, N. (2015). An updated method for isolation, purification and characterization of clinically important antioxidant lignans–sesamin and sesamolin from sesame oil. *Industrial Crops and Products*, 64, 201-208.

De, Sukumar. (2015). Outlines of dairy technology. 40th edition, Oxford University Press, New Delhi. pp. 382-466

Fukuda, Y., Nagata, M., Osawa, T., and Namiki, M. (1986). Contribution of lignan analogues to antioxidative activity of refined unroasted sesame seed oil. *Journal of the American Oil Chemists' Society*, 63(8), 1027-1031.

Gerstenmeyer, E., Reimer, S., Berghofer, E., Schwartz, H., and Sontag, G. (2013). Effect of thermal heating on some lignans in flax seeds, sesame seeds and rye. *Food chemistry*, 138(2), 1847-1855.

Gosewade, S. (2013) Studies on the physico chemical changes occurring in ghee during storage. M.Sc. Thesis. ICAR-National Dairy Research Institute (Deemed University), Karnal, Haryana, India.

Ghosh, J., Banerjee, A., Gupta, S. S., Sengupta, A., and Ghosh, M. (2014). Comparative degradation effects of sesame and soybean oil during heating using microwave irradiation. *Journal of Scientific & Industrial Research*, *73*, 547-552.

Hwang, L. S. (2005). Sesame Oil. In: Fereidooi Shahidi, editor. Bailey's Industrial Oil and Fat Products. 6th Edition, Vol.2. John Wiley and Sons, Inc. pp 537-564.

Hemalatha, S. (2007). Sesame lignans enhance the thermal stability of edible vegetable oils. *Food Chemistry*, 105(3), 1076-1085.

Hariyani, A. (2010). A study on physico-chemical qualities of market ghee. M.Sc. Thesis. ICAR-National Dairy Research Institute, Karnal (Deemed University). Karnal, Haryana, India.

Haribabu, K., Ajitha, M., and Mallavadhani, U. V. (2014). Simultaneous determination of asaranin and sesamin in piper chaba fruit by using HPTLC-MS method: effect of different extraction methods on the yield of marker compounds. *Journal of Analytical & Bioanalytical Techniques*, 5(4), DOI:10.4172/2155-9872.1000199.

IS: 3508 (1966). Indian standards, Methods for sampling and test of ghee (Butter Fat). Bureau of Indian Standards. Delhi, India: Simco printing press.

IS 548-2 (1976). Indian Standards, Methods for sampling and test for oils and fats. Bureau of Indian Standards. Delhi, India: Prabhat offset press.

Kamal-Eldin, A., Appelqvist, L. A., and Yousif, G. (1994). Lignan analysis in seed oils from four sesamum species: comparison of different chromatographic methods. *Journal of the American Oil Chemists Society*, 71(2), 141-147.

Kim, K. S., Lee, J. R., and Lee, J. S. (2006). Determination of sesamin and sesamolin in sesame (*Sesamum indicum L.*) seeds using UV spectrophotometer and HPLC. *Korean Journal of Crop Science*, 51(1), 95-100.

Li, Y., Ye, M., Liu, H., Ji, X., and Yan, Y. (2002). Characterization and analysis of Semen Cuscutae by capillary gas chromatography and gas chromatography–mass spectrometry. *Journal of separation science*, 25(4), 255-259.

Lee, J., and Choe, E. (2006). Extraction of lignan compounds from roasted sesame oil and their effects on the autoxidation of methyl linoleate. *Journal of Food Science*, 71(7), C430-C436.

Liang, M. T., Liang, R. C., Huang, L. R., Hsu, P. H., Wu, Y. H., and Yen, H. E. (2012). Separation of sesamin and sesamolin by a supercritical fluid-simulated moving bed. *American Journal of Analytical Chemistry*, 3(12A), 931.

Lee, W. J., Su, N. W., Lee, M. H., and Lin, J. T. (2013). Examination of the modified villavecchia test for detecting sesame oil. *Journal of the American Oil Chemists' Society*, 90(5), 667-674.

Mohamed, H. M. A., and Awatif, I. I. (1998). The use of sesame oil unsaponifiable matter as a natural antioxidant. *Food Chemistry*, 62(3), 269-276.

Parmar, S. (2005).Evaluation of quality of market ghee. M.Sc. Thesis. ICAR-National Dairy Research Institute, Karnal (Deemed University). Karnal, Haryana, India.

Pawar, N., Gandhi, K., Purohit, A., Arora, S., & Singh, R. R. B. (2014). Effect of added herb extracts on oxidative stability of ghee (butter oil) during accelerated oxidation condition. *Journal of Food Science and Technology*, 51(10), 2727-2733.

Rao, D. V., and Ramamurthy, M. K. (1987). Influence of the metal catalysts on the pattern of carbonyl production during the autoxidation of cow milk fat. *Indian Journal of Animal Sciences*, 57(5), 475-478.

Rao, L. V., Ranganadham, M., and Rao, B. V. R. (2004). Quality of milk and Milk products marketed in hyderabad city. Part II: chemical quality of milk products. *Indian Journal of Dairy Science*, 57(3), 171-176.

Rahman, M. S., Hossain, M. A., Ahmed, G. M., Uddin, M. M., and Yeasmin, S. (2008). Studies on the effect of antioxidants on the stability of sesame (*Sesame indicum linn.*) seed oil during storage. *Bangladesh Journal of Scientific and Industrial Research*, 43(2), 235-242.

Rangkadilok, N., Pholphana, N., Mahidol, C., Wongyai, W., Saengsooksree, K., Nookabkaew, S., and Satayavivad, J. (2010). Variation of sesamin, sesamolin and tocopherols in sesame (Sesamum indicum L.) seeds and oil products in Thailand. *Food Chemistry*, 122(3), 724-730.

Reshma, M. V., Balachandran, C., Arumughan, C., Sunderasan, A., Sukumaran, D., Thomas, S., and Saritha, S. S. (2010). Extraction, separation and characterisation of sesame oil lignan for nutraceutical applications. *Food Chemistry*, 120(4), 1041-1046.

Subrahmanyam, V.,Srinivasan, M., and Bhalerao, V.R. (1952). Adulteration of ghee and its detection. *Journal of Scientific and Industrial Research,* 11(A), 277-282.

Sharma, U. P., and Zariwala, I. T. (1978). Survey of quality of milk products in bombay. *Journal of Food Science and Technology*, 118-121.

Sharma, S. K. (2009). Studies on unsaponifiable matter of ghee (clarified butterfat) and animal body fats with a view to detect adulteration. *Ph.D. Thesis.* ICAR-National Dairy Research Institute (Deemed University), Karnal, Haryana, India.

Sharma, R. (1992). Physico-chemical properties of ghee prepared from milk adulterated with vanaspati and animal body fats. M.Sc. Thesis. ICAR-National Dairy Research Institute, Karnal (Deemed University). Karnal, Haryana, India.

Suja, K. P., Abraham, J. T., Thamizh, S. N., Jayalekshmy, A., and Arumughan, C. (2004). Antioxidant efficacy of sesame cake extract in vegetable oil protection. *Food Chemistry*, 84(3), 393-400.

Shukla, A. K., Dixit, A. K., and Singh, R. P. (2005). Detection of adulteration in edible oils. *Journal of Oleo Science*, 54(6), 317-324.

Schwertner, H. A., and Rios, D. C. (2012). Analysis of sesamin, asarinin, and sesamolin by HPLC with photodiode and fluorescent detection and by GC/MS: application to sesame oil and serum samples. *Journal of the American Oil Chemists' Society*, 89(11), 1943-1950.

Thirumala Rao, S. D., Ramachar, D., and Krishnamurthy, G. (1968). A new color reaction of sesame oil. *Journal of the American Oil Chemists' Society*, 45(6), 477-477.

Wang, C. C., Chen, S., and Wu, T. S. (2003). The facile reversed-phase HPLC resolution of tetrahydrofurofuran nucleus lignans in traditional chinese medicine: quantitative analysis of asarinin and sesamin in asari radix. *Journal of the Chinese Chemical Society*, 50(2), 261-266.

Wu, W. H. (2007). The contents of lignans in commercial sesame oils of Taiwan and their changes during heating. *Food Chemistry*, 104(1), 341-344.

Wang, L., Zhang, Y., Li, P., Wang, X., Zhang, W., Wei, W., and Zhang, X. (2012). HPLC analysis of seed sesamin and sesamolin variation in a

sesame germplasm collection in China. *Journal of the American Oil Chemists' Society*, *89*(6), 1011-1020.

Wan, Y., Li, H., Fu, G., Chen, X., Chen, F., and Xie, M. (2015). The relationship of antioxidant components and antioxidant activity of sesame seed oil. *Journal of the Science of Food and Agriculture*, 95(13), 2571-2578.

Yoshida, H., Shigezaki, J., Takagi, S., and Kajimoto, G. (1995). Variations in the composition of various acyl lipids, tocopherols and lignans in sesame seed oils roasted in a microwave oven. *Journal of the Science of Food and Agriculture*, 68(4), 407-415.