# TRIACYLGLYCEROL PROFILING AND GENOMIC DNA ANALYSIS TO ASCERTAIN THE PURITY OF GHEE

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

DOCTOR OF PHILOSOPHY
IN
DAIRY CHEMISTRY

BY

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**M. Tech. (Dairy Chemistry)**

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KARNAL-132001 (HARYANA), INDIA

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# CERTIFICATE

This is to certify that the thesis entitled "**TRIACYLGLYCEROL PROFILING AND GENOMIC DNA ANALYSIS TO ASCERTAIN THE PURITY OF GHEE**" submitted by Mr. Tanmay Hazra towards the partial fulfilment of the requirements for the award of the degree of DOCTOR OF PHILOSOPHY in DAIRY CHEMISTRY of the ICAR-NATIONAL DAIRY RESEARCH INSTITUTE (DEEMED UNIVERSITY), Karnal (Haryana), India, is a bonafide research work carried out by him under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

**(Dr. Vivek Sharma)**
**Major Advisor**
**(GUIDE)**

Dated: 09/02/2017

Dedicated
To
My Father &
Guide

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TANMAY HAZRA

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# List of Abbreviation

| | |
|---|---|
| % | Percentage |
| °C | Degree Celsius |
| Approx. | Approximately |
| AGMARK | Agriculture Produce (Grading and Marking) |
| AR | Analytical Grade |
| AFLP | Amplified Fragment Length Polymorphisms |
| B.R | Butyro-refractometer |
| B.I.S | Bureau of Indian Standards |
| CLT | Complete Liquifaction Time |
| cP | Centi poise |
| DNA | Deoxyribonucleic acid |
| (dNTP) | Deoxynucleotide |
| et al. | Et (and) alia (other) |
| FFA | Free fatty acid |
| FSSAI | Food Safety and Standards Authority of India |
| g | Gram |
| GLC | Gas Liquid chromatography |
| GBF/GF/Goat tallow | Goat body fat |
| HPLC | High Performance Liquid Chromatography |
| Hrs | Hours |
| IDF | International Dairy Federation |
| I.R | Infra red |
| L | Liter |
| Lard | Pig body fat |
| min | Minute |
| nm | Nano meter |
| O.D | Optical density |
| P.C.R | Polymerase chain reaction |
| RNA | Ribonucleic acid |
| S.D. | standard deviation |
| S.E. | Standard error |
| TLC | Thin layer chromatography |

# ABSTRACT

India is the largest milk producing country and Ghee is the second largest dairy product prepared from milk i.e. 35% of total milk production is used for preparation of ghee. Ghee is adulterated with various adulterants due to high market demand and low production. Unfortunately, the producers or the middle-men involved in the ghee trade, in their greed to have more money, tend to adulterate ghee with cheaper oils and fats like 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. FDA (Punjab) recently reported that almost 40% ghee in market of Punjab is adulterated. Literature revealed that the techniques suggested to ascertain the quality of milk fat have their own limitations and are being manipulated by the unscrupulous traders. To counter this menace of adulteration a systemic study was conducted to ascertain the purity of ghee on the basis of genomic DNA analysis and triglyceride profiling using GLC. Various conventional as well as commercial kit based protocols have been evaluated for isolation of DNA from fat samples. Yield and purity of DNA was checked by Nanodrop and quality by agarose gel electrophoresis. Amplification of isolated DNA was tested with universal primers (1&2). It was observed that modified Nucleo Spin tissue kit and QIAamp stool kit based protocol were able to isolate sufficient quantity of DNA for downstream PCR application. For developing PCR based technology for detection of animal body fat in ghee, various species specific primers were used and observed that short length primers were able to amplify DNA from fat samples. Modified Nucleo spin tissue kit based DNA isolation protocol followed by species specific amplification was able to detect 20% adulteration of body fat (Goat Tallow and Lard) in ghee whereas QIAamp stool kit based protocol detected even 10% adulteration of goat tallow and lard in ghee. Triglyceride (TG) profiling of ghee using GLC, sixteen even carbon number TGs were observed both in cow and buffalo ghee. It was observed that medium and higher carbon number triglycerides were predominant in cow or buffalo ghee. In case of body fat 50 and 52 carbon numbers triglyceride (C50 and C52 TGs) were predominant whereas in soy oil 54 carbon numbers triglyceride (TG) was predominant. As a result of adulteration of ghee with individual body fat or soy oil a significant increase in triglycerides having 50 to 54 carbons (C50, C52 and C54) was observed and summation of these (three TGs – C50+C52+C54) markers was able to detect adulteration of ghee with body fat or vegetable oil to the tune of 5%. It was also possible to detect the adulteration of ghee with body fats (Goat tallow or lard and soy oil) if they are added in combination but difficult to ascertain the type of body fat added to ghee using GLC approach.

# सारांश

भारत सबसे बड़ा दुग्ध उत्पादक देश है और 35% कुल दूध उत्पादन का उपयोग घी तैयार करने के लिए किया जाता है| बाजार की ज्यादा मांग और कम उत्पादन के कारण विभिन्न मिलावटी तत्वो के साथ घी की मिलावट की जाती है। ज्यादा पैसो के लालच में, सस्ते तेल और वनस्पति तेलों, पशु शरीर चरबी, हाइड्रोजनीकृत तेल, और कभी कभी भी गैर-खाद्य खनिज तेल के साथ कुछ मौसम के दौरान विशेष रूप से घी की मिलावट की जाती है| हाल के वर्षों में मिलावट की समस्या ने एक बहुत गंभीर आयाम ले लिया है। एफडीए (पंजाब) ने हाल ही में रिपोर्ट किया है कि पंजाब के बाजार में लगभग 40% घी मिलावटी है। साहित्य से पता चला है कि दूध में वसा की गुणवत्ता का पता लगाने के लिए सुझाई गई तकनीको की अपनी सीमाएं हैं और बेईमान व्यापारियों द्वारा इसमें चालाकी से हेरफेर किया जा रहा हैं| मिलावट के इस खतरे का मुकाबला करने के लिए और घी की शुद्धता का पता लगाने के लिए जीनोमिक डीएनए विश्लेषण और ट्राइग्लिसराइड जी.एल.सी के उपयोग के आधार पर एक प्रणालीगत अध्ययन आयोजित किया गया| विभिन्न पारंपरिक एवं वाणिज्यिक किट आधारित प्रोटोकॉल का घी और चरबी नमूने से डीएनए के अलगाव के लिए मूल्यांकन किया गया है| डीएनए की शुद्धता और उपज की जाँच नैनोड्रोप से और गुणवत्ता की जाँच आगारोज़ जेल वैद्युतकणसंचलन द्वारा की गयी| अलग डीएनए का प्रवर्धन का परीक्षण यूनिवर्सल प्राइमरों (1&2) के साथ किया गया था | यह पाया गया है कि संशोधित Nucleo-Spin ऊतक किट और QIAamp मल किट आधारित प्रोटोकॉल पीसीआर अनुप्रयोग के लिए डीएनए की पर्याप्त मात्रा को अलग करने में सक्षम थे| घी में पशु शरीर में चरबी का पता लगाने के लिए पीसीआर आधारित प्रौद्योगिकी विकसित करने के लिए, विभिन्न प्रजातियों के विशिष्ट प्राइमरों थे इस्तेमाल किया गया और पाया गया कि कम लंबाई वाले प्राइमर चरबी नमूने से डीएनए बढ़ाने में सक्षम थे| Nucleo-Spin ऊतक किट आधारित डीएनए अलगाव प्रोटोकॉल और उसके बाद प्रजातियों विशिष्ट प्रवर्धन द्वारा घी में 20% शरीर चरबी की मिलावट का पता लगाने के लिए किया गया जबकि QIAamp मल किट आधारित प्रोटोकॉल द्वारा घी में सिर्फ 10% शरीर चरबी के अपमिश्रण का पता लगाया गया|GLC का उपयोग कर ट्राइग्लिसराइड्स (टीजी) घी की रूपरेखा का एक परिणाम के रूप में, सोलह भी कार्बन नंबर टीजी गाय और भैंस के घी में दोनों मनाया गया। यह देखा गया है कि मध्यम और उच्च कार्बन नंबर ट्राइग्लिसराइड्स गाय या भैंस के घी में प्रमुख थे । शरीर चरबी के मामले में 50 और 52 कार्बन संख्या ट्राइग्लिसराइड (C50 और C52 टीजी) प्रमुख थे जबकि सोया तेल में 54 कार्बन नंबर ट्राइग्लिसराइड प्रमुख था। अलग-अलग शरीर चरबी या सोया तेल के साथ घी की मिलावट के परिणामस्वरूप 50-54 (C50, C52 और C54) ट्राइग्लिसराइड बढे हुए पाए गए| और इन मार्करों का योग (तीन टीजीS-C50 C52 C54) घी में शरीर वसा या वनस्पति तेल की मिलावट का पता लगाने में सक्षम पाया गया| अगर वे संयोजन में जोड़े जाते हैं, तो शरीर चरबी के साथ घी की मिलावट का पता लगाना भी संभव था लेकिन जी.एल.सी दृष्टिकोण का उपयोग करके घी में शरीर चरबी के प्रकार का पता ।

## CHAPTER - 1

# Introduction

# 1. INTRODUCTION

India is the largest producer of milk in the world, producing 146.3 million metric tons (MMT) in the year 2014-15 (Anonymous, 2016a). In India, Ghee is the second largest dairy product prepared from milk (Anonymous, 2016b). Ghee (from Sanskrit ghṛita meaning "sprinkled") is clarified milk fat and by far the most important product widely consumed in the Indian sub-continent since time immemorial. Its image as a natural product and its organoleptic attributes, nutritional value and functional properties make it suitable for numerous food applications. According to FSSR (2011) **"Ghee"** means the pure clarified fat derived solely from milk or curd or from desi (cooking) butter or from cream to which no coloring matter or preservative has been added. According to GAIN report (2014) it is estimated that in the year 2015-16 the production of ghee would be increased to 3 MMT to 5 MMT valued at Rs 475 billion. Over 10% of this production comes from organized sector, half of which is contributed by the co-operative sector. About 60% ghee is packed in bulk and 40% in consumer packs (Aneja *et. al*., 2002 & Upadhyay, 2014). Ghee production level varies from region to region, 57% in northern region, 9.5% in eastern, 23.5% in western and 10% in southern region (Anonymous, 2016b). 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 (Rangappa and  Achaya, 1974). Ghee performs a major and essential function as carrier of four fat-soluble vitamins viz., A, D, E, K and essential fatty-acids such as linolenic acid and arachidonic acid, apart from having rich and pleasant sensory attributes. Ghee is believed to be a coolant, capable of increasing mental power and physical appearance, curative of ulcers and eye-diseases (Rangappa and Achaya,1974). Unfortunately, the producers or the middle-men involved in the ghee trade, tend to adulterate ghee with cheaper oils and fats like vegetable oils, animal body fats, hydrogenated fats and sometimes even the non-edible mineral oils, especially during lean season to earn more money. Recently, 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. Reports have been appearing in the print and electronic media, indicating the rampant malpractices of ghee adulteration particularly in the central and northern parts of the country. It is not known as to what extent these types of malpractices of adulteration are prevailing in the ghee trade in our country and what quality of ghee is available to the consumers. Among all the adulterants, body fat is the most dangerous in Indian context, by adding animal body fats in ghee (the sacred holy food) the unscrupulous ghee traders are not only robbing the people of their money, but also playing with the religious sentiments, especially of the vegetarian section of the society. In order to ensure a genuine product to the consumer, the Government of India has prescribed the compositional standards for ghee, under FSSR (2011) and AGMARK rules (1981). However, these standards are not very much relevant in the present context and can hardly establish the type and the level of added adulterants. This may be because of wide variations in the physico-chemical makeup of milk fat owing to different factors like animal species, feeding practices and nutritional management, as well as meticulous way of adulteration being followed by unscrupulous people. Extensive survey of the literature reveals that in the past, several techniques have been employed to detect the adulterants in ghee, yet it is realized that they have their own limitations in establishing the type and the level of the adulterants. Recently, RP-TLC and RP-HPLC based methodologies have been developed to detect the adulteration of Ghee with vegetable oils. However, the detection of body fat & body fat in conjunction with vegetable oils is still a challenge. Hence, the methodology available till date is not adequate in dealing with the menace of adulteration. So, researchers are always in search of sensitive and specific methods for detection of adulteration in ghee.

Recently, it has been found that molecular marker based techniques are gaining popularity to ensure the authenticity and traceability of food products. Advances in DNA technology have led to rapid development of genetic methods for identification of origin of food, DNA offer advantages over proteins, including stability at high temperature, presence in all tissue types, and greater variation with genetic code (Bottero and Dalmasso, 2010). DNA can be analysed using techniques such as sequencing, DNA hybridization and the polymerase chain reaction (PCR), which are based on the detection of species-specific DNA sequences in food products (Hassan and Ali, 2012). In particular, PCR-based techniques have a high potential because of their rapidity, increased sensitivity and specificity (Lockley and Bardsey, 2000). Various DNA based methodologies already been proved highly sensitive and specific than other protein and immunochemistry based methods to prove the authenticity of meat, vegetable, rice, olive oil, milk and little bit in milk products like cheese and milk powder. But till date DNA based methodology has not been fully exploited for checking the purity and authenticity of milk fat based product like Ghee.

Another approach described in literature is fatty acid profiling of fats or oils, and their average values can be easily found in the literature (Bosque-Sendra *et al*., 2012). However, this information is not enough to tackle the issue of adulteration because different blends of fats in the right proportion could lead to similar fatty acids profiles. Recently, different researchers reported that fatty acid composition of milk fat depends on feed, season and geographical region, hence it is not an easy task to establish the authenticity of milk fat on the basis of fatty acid markers (Bosque-Sendra *et al.*, 2012).In another study, Rebechi *et al*. (2016) reported that milk fat adulterated with body fat at the rate of 15% could not be detected using fatty acid profiling.  For these reasons, recent trends are directly use the Triacylglycerol or popularly known as Triglyceride (Fox and McSweeney, 1998),  as compositional markers in order to characterize and ascertain the purity of fats and oils (Bosque-Sendra *et al.*, 2012). IDF&ISO (2010) derived regression equation on the basis of triglyceride profiling using GLC. However, GLC profiling of triglyceride as suggested by IDF&ISO (2010) has not been studied systematically in Indian scenario. So considering all the facts stated above, the main aim of the present study was to develop a DNA based protocol to detect the adulteration of ghee with body fats and to check the suitability of IDF&ISO (2010) method under Indian conditions.

Patel (2011) reported that pig body fat (lard) and goat body fat were very much difficult to detect in ghee and very recently Nikhil and De (2015) able to detect buffalo tallow in ghee but detection of goat tallow as well as lard in ghee is still a challenge. So we selected goat body fat as well as lard in this present study.

Therefore, the present study was proposed with the following objectives:

- **To optimize the isolation process of genomic DNA from ghee, body fat and adulterated samples of ghee.**
- **Standardization of Genomic DNA based methods for detection of body fat adulteration in ghee.**
- **To differentiate the ghee and adulterated ghee on the basis of triglyceride profile using GLC.**

## CHAPTER - 2

# Review of Literature

# 2.0 REVIEW OF LITREATURE

This review delineates the latest status of methodology being used for the detection of adulteration of milk fat with foreign fats. Old methodologies like melting point, Bomer value, Titre value have lost the relevance in the present scenario of adulteration. Therefore in the present situation the recent developed methods have been reviewed.

The literature has reviewed under the following three major heads: **2.1) Traditional methods used for detection of adulteration in milk fat, 2.2) DNA based methodology for quality control in food products, 2.3) Gas Liquid Chromatography of Triglyceride**

## 2.1) Traditional methods used for detection of adulteration in milk fat

The literature has been reviewed under the following four major heads: **2.1.1) Methods based on physical properties, 2.1.2) Methods based on chemical properties, 2.1.3) Methods based on tracer components of fats and oils, and 2.1.4) Miscellaneous methods.**

### 2.1.1) Methods based on physical properties

Physical properties of oils and fats are important criteria for judging their quality and have also been used to determine their authenticity.

Several methods, which were used to check the authenticity of ghee on the basis of physical properties, are as follows:

#### 2.1.1.1 Butyro Refractometer Reading (BR)

Refractive index and BR readings are inter-convertible (Rangappa and Achaya, 1974; Winton and Winton, 1999). The values for B.R. readings of milk fat (40-45) and vegetable oils and fats (above 50) are so wide apart (Singhal, 1980; Gunstone *et al*., 1994) that this property could be safely employed as an index for milk fat adulteration with vegetable oils and fats, except coconut oil (38-39) and palm oil (39-40). Feeding of cottonseed oil raises the B.R. reading by 5 units in case of ghee (Rangappa and Achaya, 1974 & Winton and Winton, 1999). Normally, BR reading or refractive index of oils and fats increases with the increase in unsaturation and also chain length of fatty acids. The B.R. readings of animal body fats are in the range of 44 to 51 (Singhal, 1980). Adulteration of milk fat with animal body fats (Singhal, 1973; Sharma and Singhal, 1995) and vanaspati (Sharma and Singhal, 1995) at a level of 5 to 20 percent increased its B.R. readings. Recently, some workers (Arora *et al*., 1996; Lal *et al*., 1998) have developed a simple platform test for the detection of vegetable oil (refined mustard oil) added to milk at a level higher than 10 percent of the original fat on the basis of increase in B.R. reading of the fat. Kumar (2008) noticed that the adulteration of ghee with animal body fat up to 15% level studied could not be detected by using B.R. reading.

Patel (2011) reported that vegetable oil added to cow ghee at 10 percent level and above levels could easily detected by B.R reading, Whereas, in buffalo ghee, only 15 percent level of vegetable oil could be detected. On the other hand animal body fat added to both types of ghee (cow and buffalo) could not be detected less than 15% level. In a different study Pranoti (2012) reported that using B.R reading 20% lard could be detected in cow ghee but this said method was not useful to detect lard adulteration in cow or mix ghee. Kumar (2013) reported that B.R. reading could not be used to detect body fat adulteration in ghee (cow or buffalo) less than 15% level.

#### 2.1.1.2 Opacity Test

Singhal (1980) developed an opacity test to detect the adulteration of ghee with animal body fats. He concluded that the adulteration with buffalo, goat and sheep body fats at 5 percent level and above could be safely detected by opacity test. However, the limitations of this test are that the detection of pig body fat up to 10 percent level is difficult and ghee from cotton tract area also cannot be distinguished. Test also failed to detect the body fats in ghee in the presence of vegetable oils (Singhal, 1987).

Sharma and Singhal (1996) carried out the opacity test at 25°C for pure fats (ghee, body fats and vanaspati) and adulterated ghee samples (5, 10 and 20% level) and observed almost the similar results as reported earlier by Singhal, (1980). Their study revealed that ghee took more than 35 min to become opaque, while vanaspati and lard (pig body fat) took 2 to 2.5 min and 6 to 7 min, respectively. On the other hand, tallow (buffalo and goat body fats) took very less time (<18 seconds) to become opaque. They did not find any significant difference in the opacity time of pure ghee and ghee samples adulterated with vanaspati and lard even up to 20 percent level. However, in case of adulteration with buffalo and goat body fats, the opacity times were significantly reduced, thereby making the detection possible. Here also, mode of adulteration (whether added directly to ghee or through milk) did not show marked differences.

### 2.1.1.3 Critical Temperature of Dissolution (CTD)

Critical temperature of dissolution (temperature at which turbidity appears on gradual cooling of the fat dissolved in a warm solvent or solvent mixture) is a characteristic of a particular fat which depends upon the nature of the solvent, nature and amount of most insoluble glycerides (usually tri- saturated glycerides) present in a fat as well as the mutual solubilizing power exerted on these glycerides by the soluble glycerides (Rangappa and Achaya, 1974; Boghra *et al.*, 1981). Bhide and Kane (1952) observed the CTD values for ghee and vanaspati in the range of 39 to 45°C and 62 to 72°C, respectively, employing a 2:1 (v/v) mixture of 95 percent ethanol and iso-amyl alcohol, and reported that gross adulteration of ghee with vanaspati could easily be detected. Delforno, (1964) reported the CTD values for butter as 50.5 to 57.5°C (Average 54°C), for coconut oil as 28 to 41°C (Average 33°C) and for other oils and fats as 67 to 82.5°C (Average 71-78°C). Similarly, the presence of body fats in ghee was detected by employing either a single solvent such as absolute alcohol (Delforno, 1964) or a solvent mixture of 95 percent ethyl alcohol and iso-amyl alcohol in the ratio of 2:1 (Bhide and Kane, 1952). The CTD test seems to be simple, but its efficacy is greatly affected by the free acidity (FFA) and rancidity (peroxides).

Kumar (2013) & Upadhyay (2014) reported that CTD value ($^{o}$C) for pure cow ghee ranged from 51.6 to 54.6 with an average of 53.3, whereas that for pure buffalo ghee ranged from 52.4 to 56.2 with an average of 54.3. Kumar (2013) further reported that adulteration of cow as well as buffalo ghee with vegetable oil (soybean oil) and animal body fat (buffalo body fat) individually at 5, 10 and 15 percent levels and in their combinations at 5+5 (10), 10+10 (20) and 15+15 (30) % levels increased the CTD value and this increase was dependent upon the amount of adulterants added to the pure cow and buffalo ghee. Higher the level of adulterant added, greater was the increase in the CTD value of ghee samples. The author also reported that addition of animal body fat (buffalo body fat) caused slightly higher increase in the CTD value of ghee as compared to vegetable oil (soybean oil). Upadhyay (2014) also reported that using CTD value only 15% adulteration could be detected.

#### 2.1.1.4 Fractionation of milk fat

Fractionation is a thermally controlled process (with or without solvent) in which the milk fat is subjected to a specific temperature/time profile to allow a portion of milk fat to crystallize. The crystals are then physically separated from the liquid fraction using vacuum filtration, pressure filtration, centrifugation etc. Single step melt crystallization or dry fractionation of milk fat into lower and higher melting fractions reveal that the lower melting fractions of milk fat contain somewhat greater levels of unsaturated acids and the fat constants for these fractions show a lower saponification number, a higher iodine value, a higher refractive index, and, of course, a much lower melting range (Gandhi *et al*., 2015).

Fractionation of fat with or without the use of solvent under suitable conditions of time and temperature combinations, followed by examination of fractions thus obtained has been exploited by some workers as a tool to detect foreign fats in milk fat. Bhalerao and Kummerow, (1956 a & b) were able to detect, the presence of foreign fats at 10 percent level in milk fat.

Latif and Mazloum, (1969 a & b) carried out fractionation approach and able to detect of 10 percent vegetable fat in milk fat. Farag *et al.* (1983) carried out the fractional approach of milk fat followed by GLC of their fatty acid profile and able to detect vegetable oil @ 10.

Panda and Bindal (1998) studied the crystallization behaviour at 17°C of fat dissolved in a solvent mixture of acetone and benzene (3.5:1) and reported that ghee, ghee adulterated with body fats (10%) and ghee adulterated with vegetable oils and fats (10% level) took 19 min, 3 to 15 min and 22 to 23 min to crystallize. They concluded from the study that even low level adulteration of animal body fats and vegetable oils and fats could be detected in ghee. However, for cotton tract ghee, Panda and Bindal (1998) carried out crystallization test at 25°C instead of 17°C. But, the test could not distinguish between cotton tract ghee and the ghee adulterated with cow body fat at 5 percent level.

Upadhyay (2014) reported that solvent fractionation method coupling with BR was very efficient for detection of body fat in both cow and buffalo ghee @20% level but less than 20% level could not be detected in this above said method. In same study the author carried out dry fractionation at temperature combination of 15ºC for 70-80 minutes followed by temperature time combination of 10ºC for 50-60 minutes to get first solid fraction and last liquid fraction of pure and adulterated ghee and suggested that BR reading of last liquid fraction even 10% level of body fat adulteration could be detected.

### 2.1.1.5 Spectroscopic Methods

#### 5.a Tests Based on Visible Spectroscopy

Jha, (1981) applied visible spectroscopy for the detection of Cheuri (*Madhuca butyracea*) fat in ghee, a common adulterant in Nepal. Pure ghee showed no absorption band in visible range (600-700 nm), whereas, Cheuri fat showed an absorption band with maxima between 640 and 680 nm. Even 5 percent Cheuri fat content added to ghee could be detected in this range. In another study Kumar *et al*. (2010) reported that visible spectroscopic method was not so much useful for detection of adulteration in ghee as there was no significant difference absorption pattern between pure ghee as well as adulterants and adulterated ghee.

### 5.b Tests Based on Ultraviolet (UV) Spectroscopy

Rego *et al.* (1963) examined the UV spectra of the fats from butter, margarine and their mixture after dissolving in hexane, and reported that all the samples of genuine butter, but not the margarine, gave a peak with a maximum at about 232 nm. Singhal (1973) and Sharma (1989) scanned the UV spectra of cow ghee, buffalo ghee and animal body fats (buffalo, goat, pig and sheep) between 200 to 320 nm after dissolving the fats in n-hexane and observed a maximum absorption between 220 to 230 nm. However, cow ghee showed another small maximum at 270 nm. These workers also examined the UV spectrum of unsaponifiable matter extracted from ghee and animal body fats between 200 to 320 nm and observed an absorption maxima between 215 to 220 nm for both fats. However, the ghee samples showed a second maxima at 270 nm, which was shifted to 260 nm (Singhal, 1973) or to 280 nm (Sharma, 1989) in case of animal body fats. But, on this basis, adulterated ghee could not be differentiated from pure ghee (Sharma, 1989; Kumar, 2003).

Rakesh (2016) studied that the 2nd order derivative UV spectroscopy did not offer any help in detecting the adulteration of ghee, because no characteristic differences were observed in the absorption behavior of different oils and fats including milk fat studied in the UV (200-400 nm).

### 5.c Tests Based on Infra-Red (IR) Spectroscopy-

Infra-red absorption has been extensively used in the analysis of lipids especially for cis- and trans- isomers. Unsaturated fatty acids of natural vegetable oils and fats are in cis- configuration and are isolated (non-conjugated). Partial hydrogenation or oxidation may result into formation of trans-isomers. Animal and marine fats may also contain small amounts of natural trans-isomers (Kirk and Sawyer, 1999). Bovine milk fat contains a low level (5%) of trans fatty acids in comparison with hydrogenated vegetable oils, in which the value may be as high as 50 percent due to non-stereospecific hydrogenation (Fox and McSweeney, 1998). For demonstrating the presence of hydrogenated fats in milk fat, some workers (Bartlett and Chapman, 1961; Firestone and Villadelmar, 1961) applied IR spectrophotometry and observed that the absorption maximum at 10.36 µ gets increased by the addition of hydrogenated fats containing iso-oleic acids (trans-octadecenoic acid).

Francesco *et al.* (1971) studied the compositional differences in the unsaponifiable matter of butterfat and synthetic fat by IR spectroscopy of the fractions obtained by thin layer chromatography on silica gel and observed that unsaponifiable matter of butterfat contained a polyene fraction which was absent from that of synthetic fat. They also carried out the fractionation of the unsaponifiable matter from the two fats by column chromatography on alumina and on the basis of differences in their IR absorption pattern; they reported that adulteration of butterfat with synthetic fat at 10 percent level could be detected.

Konevets *et al.* (1987) studied the cis-trans configurations of individual fats (milk fat, animal body fat, vegetable fat and hydrogenated fat) and their mixtures using IR spectroscopy, and reported that the additions up to 10 percent of animal, vegetable and hydrogenated fats to milk fat could be detected. Sato *et al.* (1990) used near IR spectroscopic method for the detection of as little as 3 percent foreign fat in milk fat. Sharma, (1989) scanned the IR spectra of cow ghee, buffalo ghee, animal body fats (Buffalo, goat, sheep and pig) and ghee adulterated with body fats in the 4,000 to 600 cm$^{-1}$ region and observed distinct differences between body fats and ghee in the region of 1300 to 1180 cm$^{-1}$ and 1120 to 1100 cm$^{-1}$, respectively. Body fats showed the presence of 5 to 6 bands, while ghee showed only two bands. Ghee samples adulterated with body fats also showed 3 to 6 extra bands. Unsaponifiable matter extracted from the ghee, body fats and adulterated ghee samples also exhibited the similar pattern of bands as reported above for the whole fat. He concluded that the differences in IR spectrum of ghee and body fats could be used to detect adulteration of body fats with ghee at 10 percent level. Recently, Kumar (2003) reported that on the basis of increased level of trans isomers in ghee, as low as 5% of vanaspati added to ghee could be detected. Sushil (2015) reported that using FT-IR band pattern analysis palm oil could not be detected in ghee as both ghee and palm oil had same absorption pattern. Very recently Bency *et a*l. (2016) differentiate cow and buffalo ghee using FT-IR. In a different study Upadhyay *et al.* (2016) reported that 1% goat tallow could be detected in Ghee by FT-IR spectroscopy analysis followed by chemo metrics analysis of wavelength, but they also concluded that this method was only tried in lab samples but not tried in field samples to prove the efficacy of this method.

### 5.d Other spectroscopic methods

Koca *et al.* (2010) studied temperature-controlled attenuated total reflectance-mid-infrared (ATR-MIR) spectroscopy combined with multivariate analysis as a simple and rapid method for the determination of butter adulteration with vegetable oil. Commercial samples of butter fat were adulterated with margarine fat at levels ranging from 0% to 100% (v/v). Partial least square regression (PLSR) models gave standard error of cross-validation (SECV) of <1.2% (v/v) and correlation coefficients (r) > 0.99. Excellent predicting capabilities were obtained using an external validation set consisting of butter adulterated with margarines at ratios of 2.5%, 13%, and 45%. Additionally, infrared spectroscopy provided distinctive bands that allowed discrimination of butter and margarine samples by forming well separated clusters for the different products evaluated.

Garcia *et a*l. (2012) used MALDI-TOF mass spectroscopy and reported that during adulteration of milk fat with vegetable oil MALDI-TOF MS spectra data changed due to change in the triacylglycerol characteristic, this phenomena due to the illegal addition of non-milk fat to milk is shown to mostly increase the relative abundance of the ion clusters centred on charge by mass ratio of triacylglycerol.

Nataskatsane *et al*. (2013) detected vegetable oil in milk fat even upto 5% level using Flourosensene spectroscopy. Using three fluorophores *viz* tryptophan, tocopherols, and riboflavin as biomarkers, they were able to differentiate pure milk fat and milk fat adulterated with vegetable oil.

Nikhil and De (2015) used derivative spectroscopy to detect adulteration of tallow in ghee and they were reported to 20% level of detection of buffalo tallow in pure ghee.

#### 2.1.1.6 Differential Thermal Analysis (DTA) and Differential Scanning Calorimetry (DSC)

DTA and DSC are both closely related thermo-analytical techniques which measure the physical properties such as phase transition and specific heat of foods as a function of temperature. DTA measures the difference in temperature ($\Delta t$)

between a sample and an inert reference material as a function of temperature. In DSC thermograms, the area delineated by the output curve is directly proportional to the total amount of energy transferred in or out of the sample.

Lambelet *et al.* (1980) observed that the crystallization and melting behaviour of buffalo milk fat was perceptibly different from that of cow milk fat, the former generally beginning to solidify and melt at higher temperature, exhibiting a higher solid fat content at a particular temperature in the melting range using DSC technique. DTA and DSC have been used by different investigators for the detection of foreign fats in milk fat. Antila *et al.* (1965) detected 5 percent coconut fat, cocoa fat and hardened vegetable fat in butterfat by DSC based on the differences in the shape of melting curves. However, tallow, lard and vegetable oil added at 5 percent level in butterfat could not be detected by this method. Roos and Tuinstra (1969) using DTA showed that addition of 5 to 10 percent of beef tallow in butterfat changed the solidification curve as solidification started earlier and showed two distinct minima in the curve. Lambelet *et al.* (1980) detected goat body fat (more than 10%) in ghee by DTA technique on the basis of differences in melting diagram and crystallization patterns of goat body fat and ghee. Using DSC, detection of foreign fats like pig and buffalo body fats (Lambelet and Ganguly, 1983) and chicken fat (Coni *et al.*, 1994) in milk fat was reported. The method, however, failed to detect coconut oil, cotton tract ghee and other animal body fats.

Upadhyay *et al.* (2016) reported that cow ghee added with goat body fat @5%, 10% and 15% had different crystallization and melting curves from pure cow ghee, hence the author reported to detect 5% level of goat body fat in cow ghee.

### 2.1.2 Methods Based on Chemical Properties

Several chemical methods based on Physico-Chemical Constants, triglycerides, un-saponifiable matter, and specific tests using gas liquid chromatography (GLC), thin layer chromatography (TLC), paper chromatography, etc. have been used to characterize the various fats and oils with a view to check the purity of milk fat.

#### 2.1.2.1 Tests Based on Physico-Chemical Constants

Certain well-known physical and chemical constants have been derived for the purpose of characterization of oils and fats. Among those constants three determinations, the Reichert-Meissl value, the Polenske value and the Iodine value, measure certain specific constituents of milk fat while other two, the Saponification value and Butyro-refractometer reading, give an overall average nature of the constituent fatty acids present (Rangappa and Achaya, 1974). These physico-chemical constants are described briefly in the following sections:

##### 2.1.2.1.1 Reichert-Meissl *(*RM) value

RM value is the number of millilitre of 0.1 N alkali solution required to neutralize the steam volatile, water soluble fatty acids distilled from 5 g of fat under specified conditions. This constant for milk fat is quite significant since it is primarily a measure of butyric ($C_{4:0}$) and caproic ($C_{6:0}$) acid. RM value for milk fat ranges from 17 to 35, which is well above the value (generally 1) for all other fats and oils except coconut oil and palm kernel oil for which the value ranges between 4 to 8 (Singhal, 1973 & 1980). Feeding of cottonseed to milch animals lowers the RM value of ghee by 5 to 6 units (Rangappa and Achaya, 1974). Singhal (1973) in their study on the physico-chemical properties of different layers of ghee obtained after one month storage at 30 to 35°C reported that the liquid layer exhibited a higher RM value than solid layer.

Patel (2011) reported that RM-value was higher in buffalo ghee as compare to cow ghee. The author also reported Vegetable oils and animal body fats showed negligible RM values as against high RM values observed for cow and buffalo ghee and by using this technique higher than 10% level of vegetable oil adulteration could be detected in ghee.

Pranoti (2012) reported that pure cow adulterated with body fat 10% level and pure buffalo ghee body fat adulteration 20% level could be detected by RM-value analysis.

Rakesh (2016) studied that adulteration of pure cow ghee with soybean oil at 5% level or above, whereas animal body fats individually at 10% or above could be detected by using RM value. The adulteration of buffalo ghee with all the adulterants individually at 20% level or above could be detected by using RM value.

#### 2.1.2.1.2 Polenske value

Polenske value denotes the number of millilitre of 0.1 N alkali solution required to neutralize the steam volatile and water insoluble fatty acids distilled from 5 g of fat under specified conditions. This value is substantially a measure of caprylic ($C_{8:0}$) and capric ($C_{10:0}$) acid. The Polenske value for milk fat ranges from 1.2 to 2.4. This value for other oils and fats (Singhal, 1980; Winton and Winton, 1999) is also low (less than 1) except the coconut oil (15-20) and palm kernel oil (6-12). Feeding of cottonseeds to milch animals reduces the Polenske value of ghee by 0.3 to 0.7 units (Rangappa and Achaya, 1974). Singhal (1973) reported that the liquid portion of ghee obtained on storage at 30 to 35°C for one month showed higher Polenske value than the solid portion. Patel (2011) reported that Polenske value overall range was 1.30 to 1.80 for both pure cow and buffalo ghee and when ghee added with more than 15% body fat the range deviated to 1.28 as compare to normal range. In another study Pranoti (2012) reported that Polenske value was not suitable to detect adulteration in ghee with body fat at any level. Same type of observation was recorded by Rakesh (2016), who reported that Polenske value could not be used to detect upto 20% level of adulteration in ghee.

#### 2.1.2.1.3 Iodine value

Number of grams of iodine absorbed by 100 g of fat under specified conditions represents the iodine value. This constant is a measure of unsaturated linkages present in a fat. The iodine value for milk fat ranges from 26 to 35, which is low in comparison to most of the other fats & oils (Singhal, 1973). Animal body fats show slightly higher iodine value ranging from 36 to 49. Whereas, for vegetable oils, the value is very high (74-145) except coconut oil (6-10) and palm kernel oil (10-18). For hydrogenated fats, it lies in the range of 70 to 79. Feeding of cottonseed raises the iodine value of ghee up to 10 units (Rangappa and Achaya, 1974). Liquid fraction obtained as a result of ghee storage at 30 to 35°C for one month showed higher iodine value than solid fraction (Singhal, 1973).

Upadhyay (2014) studied that using iodine value 15% ground nut oil could be detected in buffalo ghee whereas 5% ground nut oil could be detected in cow ghee. In another study Gandhi *et al*. (2015) reported that solvent fractionation couple with iodine value analysis of liquid fraction able to detect 30% level of sheep body fat in ghee.

### 2.1.2.1 .4 Saponification value

Saponification value which denotes the number of milligrams of KOH required to saponify one gram of fat gives an indication of average molecular weight of fatty acids present. For milk fat, animal body fats, vegetable oils and hydrogenated fats, the value ranges from 210 to 233, 192 to 203, 170 to 197 and 197 to 199, respectively. Coconut oil and palm kernel oil show higher saponification value ranging from 243 to 262 (Jenness and Patton, 1969; Singhal, 1980). Feeding of cottonseeds to milch animals lowers this value by 7 units (Rangappa and Achaya, 1974). Liquid portion of ghee separated on storage at 30 to 35°C for one month showed higher saponification value than solid portion (Singhal, 1973).

Several earlier workers (Achaya and Banerjee, 1946; Velu, 1971) have reported that physico-chemical constants failed to detect the adulteration of milk fat with beef tallow, refined cottonseed oil, hydrogenated oils and even coconut oil separately or in mixture even up to 10 percent level. Singhal (1973 & 1980) also employed the physico-chemical constants for detecting the animal body fats (buffalo, goat, sheep and pig) added to buffalo and cow ghee and reported that the values for Reichert-Meissl, Polenske, and BR indices remained within the legal limits for normal ghee, when adulterated with animal body fats at 20 percent level. However, when adulteration was done at 50 percent level, the values remained within the legal limits set for cotton tract ghee. Sharma and Singhal (1995) also confirmed the above findings using body fats (buffalo, goat and pig) and vanaspati ghee irrespective of mode of adulteration whether added directly to ghee or through milk.

### 2.1.2.2 Tests Based on Gas Liquid Chromatography (GLC) of Fatty Acids

Milk fat derived from ruminant animals, contains an exceptional number and variety of fatty acids from 4:0 to 26:0 (saturated) and from 10:1 to 22:5 (unsaturated). Body fats like tallow and lard contain mostly palmitic (16:0), stearic (18:0) and oleic acid (18:1), while vegetable oils consist mainly of palmitic, stearic, oleic and linoleic (18:2) acids. Coconut oil is the best known exception, containing lauric (14:0) and myristic (16:0) acids in very large amount (Rangappa and Achaya, 1974).

The GLC technique was employed by several workers for the detection of milk fat adulteration through determining the ratios of different fatty acids. Some workers (Francesco and Avancini, 1961; Boniforti, 1962) employed GLC technique and reported that milk fat sample with a ratio of $C_{12:0}$ / $C_{10:0}$ fatty acids >1.6 or $C_{4:0}/C_{6:0}+C_{8:0}$ fatty acids >1.8 was considered to be adulterated with margarine, coconut oil or tallow or pig body fat trans-esterified with butyric acid, while other workers (Provvedi and Cialella, 1961) suggested ratios of other fatty acids such as $C_{4:0}$ / $C_{12:0}$ > 3, $C_{18:1}$ / $C_{18:0}$ > 2 and $C_{12:0}$ / $C_{10:0}$ >1 for the detection of vegetable oils in butterfat. Similarly, many other workers used the different fatty acids ratios for checking the adulteration of milk fat with vegetable oils, margarine, beef tallow, lard, goat body fat, substituted fats, synthetic fats, etc. (Toppino *et al*., 1980; Ulberth, 1994). Farag *et al*. (1983) determined the fatty acid profile of 3 fractions separated by fractional crystallization from cow and buffalo ghee adulterated with lard and margarine at various levels and reported that the amounts of 16:0, 18:0 and 18:1 acids were significantly changed with different adulteration levels and can be used as a marker to detect the admixture. Singhal (1987) reported that on GLC analysis of saturated triglycerides (STGs), ratio of fatty acids, viz., $C_{15:0}$ / $C_{17:0}$ and $C_{16:0}$ / $C_{18:0}$ was lower in ghee samples adulterated with body fats even in the presence of vegetable oils. Sharma and Singhal (1996) analyzed the buffalo ghee samples adulterated with body fats (buffalo, goat, pig) and vanaspati at 20 percent level and noted that short and medium chain fatty acids decreased while long chain fatty acids increased on adulteration. Panda and Bindal (1998) employed this technique for the detection of adulteration in ghee with vegetable oils at level as low as 5 percent using $C_{18:2}$ or $C_{22:1}$ as marker acid. Kumar (2003) found that the adulteration of ghee with body fats or vanaspati at 15 percent level and above, could be detected using different fatty acid ratios like $C_{14:0}/C_{16:0}$ and $C_{18:0}/C_{18:1}$ whereas, presence of even 5 percent vegetable oils (sunflower oil, soybean oil and groundnut oil) in ghee could be detected easily, using fatty acid ratios such as $C_{14:0}/C_{18:2}$, $C_{16:0}/C_{18:2}$, $C_{18:0}/C_{18:2}$, as well as linoleic acid ($C_{18:2}$) as a marker.

Upadhyay (2014) reported that ratio of fatty acid C10:0/C18:2 could be a efficient marker for detection of goat body fat in ghee and even 15% of goat body fat could be detected by using this parameter and this said author also reported to detect ground nut oil in ghee @5% using linoleic acid as marker.

Kumar *et al*. (2015) claimed to detect 5 % level of animal fat in ghee (cow or buffalo) using GLC as oleic acid marker. Rebechi *et al*. (2016) found that fatty acid analysis combine with regression analysis was a very authentic tool to ascertain the purity of milk fat and these author able to detect 15% level of body fat in pure milk fat.

### 2.1.2.3 Tests based on the nature and content of unsaponifiable constituents

Sterols and tocopherols are the two most important constituents of USM, which have been used to detect the vegetable fats in milk fat by using various techniques like GLC, TLC, paper chromatography, etc.

#### 2.1.2.3.1 Tests Based on Sterols

Sterols which represent maximum share of the USM range from 0.24 to 0.5 percent in butterfat, 0.03 to 0.14 percent in body fats and 0.03 to 0.5 percent in vegetable oils (Kumar *et al*., 2002). Cholesterol is the characteristic sterol of animal fats, while sterols from vegetable sources consist of a mixture collectively called as phytosterols and include ß-sitosterol, stigmasterol, campesterol, brassicasterol, etc. Low concentration of cholesterol is also reported in the sterol fractions of vegetable oils and fats (Kirk and Sawyer, 1999). In addition to cholesterol, milk fat contains traces of lanosterol, dihydrolanosterol and ß-sitosterol (Webb *et al*., 1987). Vegetable fats contain the sterols mainly in the ester form, while animal body fats contain mostly the free form (Rangappa and Achaya, 1974).

The sterols can help to distinguish between fats of animal and vegetable origin, since the melting point of cholesterol acetate (112.76-116.40°C) is substantially lower than that of the acetates of any of the phytosterols (126-137°C). Adulteration of milk fat with vegetable oils is confirmed when melting point of sterol acetate fraction is more than 117°C (IDF, 1965; Rangappa and Achaya, 1974).

A circular paper chromatographic method based on the difference in the behaviour of USM isolated from fats in ghee using a solvent mixture of methyl alcohol: petroleum ether: water (80:10:10; v/v) was developed by Ramachandra and Dastur (1959) who reported that the spot of USM of ghee moved as a whole along with the solvent front, while that of ghee adulterated with animal body fats at 5 percent level or vanaspati at 10 percent level did not move at all when observed under UV light or when exposed to iodine vapours. However, ghee from cottonseed fed animals behaved like adulterated ghee. Sharma (1989) applied paper chromatography to the USM of ghee, body fats and their mixture, and observed that

USM from ether soluble fractions of ghee adulterated with 10 percent buffalo body fat exhibited two spots. However, pig body fat in ghee could not be detected.

IDF (1965) recommended a TLC method for the detection of vegetable fats in milk fat based on the appearance of a small band of ß-sitosterol acetate in addition to the major band of cholesterol acetate using reversed phase system consisting of undecane / acetic acid-acetonitrile saturated with undecane. Ramamurthy *et al*. (1967) using thin layers of $CaCO_3$ and soluble starch (10 g + 4 g) impregnated with liquid paraffin and a solvent system consisting of methanol:acetic acid:water (20:5:1; v/v) as a developer reported that the presence of cottonseed oil, groundnut oil, sesame oil and hydrogenated fats at 10 to 13 percent level and coconut oil at 25 percent level in ghee could be detected on the basis of $R_f$ values of 0.53 and 0.44 for cholesterol and phytosterols, respectively. Parodi (1972) differentiated inter-esterified fat (a mixture of tributyrin, beef tallow and coconut oil) from milk fat by examining the free and esterified sterols using solvent mixtures of hexane: di-isopropyl ether in the ratio of 80:20 and 93:7 (v/v), respectively, on glass plates coated with silica gel G. Inter-esterified fats contained mainly sterol esters of high molecular weight fatty acids, whereas milk fat contained cholesterol mainly (90-95%) in the free form. A simple TLC method was developed by Chourasia *et al*. (1994) by spotting USM on Silica gel G coated plates using chloroform : acetone : acetic acid (90:10:1; v/v) as the developing solvent. Based on the appearance of a characteristic violet spot in adulterated ghee observed at $R_f$ 0.55 to 0.60, Kokum (*Garcinia indica*) fat in ghee up to a level of 5 percent could be detected. Sharma (1989) carried out TLC of USM of ghee and animal body fats using hexane : ether : glacial acetic acid : ethyl alcohol (25:20:5:1, v/v) as the solvent system and reported that ghee samples adulterated with 10 percent body fats resulted in the appearance of an extra spot due to dihydrocholesterol present in body fats. TLC of free and esterified sterol digitonides could detect adulteration of ghee with 10 percent body fats, while TLC of trimethyl silyl ethers (TMS) from USM failed to detect ghee adulteration. Recently Anupama *et al.* (2013) and Upadhyay (2014) reported that using RP-TLC (Reverse phase TLC) even upto 1% vegetable oil could be detected in milk fat or ghee using ß-sitosterol as biomarker. In another study using HPLC (High Performance Liquid Chromatography) it was reported that cholesterol and β-sitosterol as markers in the unsaponifiable could be used efficiently to detect even 1% vegetable oil in ghee (Anupama *et al.,* 2015).

Using GLC technique, ß-sitosterol has been shown to be an index of vegetable fat addition (Colombini *et al*., 1978; Homberg and Bielefeld, 1979), however, by this method, addition of body fats cannot be detected as body fats also have cholesterol.

### 2.1.2.4 Tests based on whole fat/triglycerides

Differences in the chemical composition of triglycerides have also been extensively used as a basis for the detection of adulteration of milk fat with foreign fats. Based on their fatty acid composition, the possible number of triglycerides in milk fat is calculated to be more than 1,300 (Barron *et al.*, 1990). Several methods based on paper chromatography, thin layer chromatography (TLC) and gas liquid chromatography of triglycerides, employed to detect the adulteration of milk fat, have been described in the following sections.

#### 2.1.2.4.1 Tests based on paper chromatography of triglycerides

Ramachandra and Dastur (1960) standardized a circular paper chromatographic technique for the detection of common ghee adulterants, like vanaspati and animal body fats by spotting 50 percent solution of fats in $CCl_4$ on the paper chromatogram using a solvent system of ethyl alcohol: iso-amyl alcohol: $CCl_4$ (35:65:10; v/v) and observed that normal ghee sample moved well away from the origin, whereas cottonseeds fed buffalo ghee did not move and behaved like adulterated ghee. Adulteration of ghee with vanaspati (10%) and body fat (5-10%) could be detected using this technique.

Rego and Garcia-Olmedo (1963) employed one dimensional paper chromatography for the butter, margarine and their mixtures and reported that adulteration of butter with margarine could be detected from the size of spot due to butyric acid of hydrolyzed fat. Singhal (1973) employed circular paper chromatography using ethyl alcohol (95%) and iso-amyl alcohol in 1:1 ratio as solvent mixture for the detection of ghee adulteration with body fats (goat, sheep, pig and buffalo). Samples dissolved in $CCl_4$ solution were spotted on filter paper. Pure cow or buffalo ghee moved with the solvent giving a single spot at the solvent front, whereas ghee samples adulterated with any of the body fats (buffalo, goat and sheep) at 5 percent level showed streaking from the point of origin. Adulteration with pig body fat (10% level) could not be detected by this method. Cotton tract ghee behaved similar to body fat samples. Singhal (1987) reported that body fats and ghee samples adulterated with body fats at 10 percent level showed streaking even in the presence of vegetable oils, but the method failed when vegetable oils alone were added. Sharma (1989) employed ascending paper chromatography using dichloromethane: isopropanol: glacial acetic acid (80:30:20; v/v) as solvent system to ghee and body fats (goat, sheep and pig) and reported that adulteration of ghee with buffalo and goat body fats below 10 percent level and pig body fat above 10 percent level could be detected even in the presence of cotton tract ghee.

Paper chromatography of whole fat was employed by Pranoti (2012) for detection of foreign fats in ghee this said author used two solvent systems i.e dichloromethane: iso-propanol: glacial acetic acid (80:30:20) and ethyl alchol: iso-amyl alchol: carbon tetrachloride (35:65:10), and thus concluded that these two solvent system failed to detect adulteration in ghee.

### 2.1.2.4.2 Tests Based on Thin Layer Chromatography of Whole Fat

Hendrickx and Huyghebaert (1968 &1970) employed TLC on Silica gel plate, using petroleum ether: diethyl ether: formic acid (60:40:1.5% v/v), for the detection of inter-esterified fats in butterfat by identifying the monoglycerides formed during the preparation of substitution fats. As little as 2.5 percent substitution fats could be detected by the appearance of a clear band of monoglycerides. Singhal (1973) applied TLC on Silica Gel-G plate using acetone: methanol: glacial acetic acid (70:30:2; v/v) as the solvent system and reported that ghee and body fats behaved in a similar way exhibiting streaking. Adulterated samples even at 10 percent level did not show any differential behavior.

Sebastian and Rao (1974) detected the presence of vegetable oils and fats in ghee (butterfat) to the extent of even 5 percent by employing TLC technique using a self-coated silica gel glass plate and solvent mixture consisting of cyclohexane, ethyl acetate and water (600:200:1) as developing solution on the basis of appearance of bands more than the specific bands of pure ghee.

### 2.1.3 Methods based on tracer components of fats and oils

Tracer components can be defined as those compounds which are present in adulterant oils and fats, either naturally or by deliberate addition, but absent in pure ghee. Addition of some tracer component in the likely adulterant of ghee has been suggested as a rapid and reliable tool to identify them in milk fat. A tracer can be a latent colour which is not detectable visually, but get identified by its colour reaction with certain chemicals or a colouring matter (natural or synthetic) which may impart direct colouration distinct from that of the natural colour of butterfat.

Among tracers, in India, sesame oil is added (5% by weight) to vanaspati according to food laws (FSSR, 2011) for its detection in ghee by Baudouin test. The method is based on the development of a permanent crimson colour due to the reaction between furfural and sesamol formed by the hydrolysis of sesamolin (present in sesame oil) in the presence of concentrated HCl. Use of hydrofuramide (Kapur *et al*., 1960) or P-hydroxy benzaldehyde (Sharma, 1989) has also been suggested in place of furfural for this test. Another tracer is tannins which are assumed to be naturally present as impurities in palm oil. Ghee samples adulterated with palm oil give purssian blue colour with potassium ferricyanide and ferric chloride reagent and based on this reaction. Bector and Sharma (2002) have reported that palm oil added to ghee can be detected at the level of 5 percent. However, limitation of this method is that the ghee samples having BHA as antioxidant also give positive test.

Gamma oryzanol, a natural tracer, having antioxidant and cholesterol lowering properties, is found to be present in the rice bran oil. It was revealed to be a mixture of phytosteryl ferulates comprising cycloartenyl ferulate, 24- methylenecycloartenyl ferulate, and campesteryl ferulate as major components. Crude rice bran oil contains ≤2 percent (v/v) oryzanol. This compound has been indicated as a marker of rice bran oil in other edible oils (Singhal, 1987). Apart from rice bran oil, gamma oryzanol is also present as natural component of corn and barley oils. On the basis of presence of gamma oryzanol, some methods have been developed recently to detect it in other edible oils. Shukla *et al.* (2004 & 2005) reported a colorimetric method for detection of rice bran oil in other vegetable oils. However, no such study has been done on the detection of rice bran oil in ghee. Kumar (2008) developed a colorimetric method based on the gamma oryzanol that could detect rice bran oil even at 2% level

### 2.1.4 Miscellaneous methods

In this section, methods such as to detect mineral oil and cotton seed oil, and methods based on enzymatic hydrolysis, etc., are described in brief.

#### 2.1.4.1 Tests for mineral oils

Adulteration of common edible oils and fats including milk fat with cheaper mineral oils, such as paraffin oil, heavy and light fuel oil, petroleum jelly, etc., has become a widespread phenomenon because of the price difference. Mineral oils also referred to as white oils are of different thickness and refractive indices. Unlike oils and fats, mineral oils are not saponifiable by alkali. This characteristic behavior of mineral oils has been used as the basis for their detection in edible oils and fats. Venkatachalam and Sundaram (1957) could detect the presence of even 1 percent of mineral oil in ghee by saponifying the test sample (1 ml) with aqueous potash followed by addition of alcohol (50%) and thorough shaking. Appearance of turbidity indicated the presence of mineral oil. Silverberg (1962) isolated the mineral oil from adulterated ghee sample by adsorbing the latter on alkaline alumina column followed by elution with light petroleum. Presence of mineral oil in eluted material was confirmed by infra-red peaks at 3.40, 6.82 and 7.25 μ.

Besides the above reports of mineral oil detection in ghee, few reports have also appeared for their detection in vegetable oils and fats using turbidity test. Using

Holde's test, the presence of as little as 0.3 percent of mineral oil in a fat can be detected by saponifying 10 drops of test sample (1 ml) with 5 ml of 0.5 N ethanolic potassium hydroxide solution and adding 5 ml of water to the hot soap solution and noting the appearance of turbidity (Winton and Winton,1999). Kumar *et al.* (2003) have also reported the detection of liquid paraffin added to ghee at the rate of 0.5 percent and above using Holde's test as described by Winton and Winton (1999).

### 2.1.4.2 Tests based on the content of specific fatty acids

Certain specific fatty acids such as butyric acid, erucic acid, iso-valeric acid, iso-oleic acid, cyclopropenoic acids, etc. which are either characteristic or absent in milk fat or present in less quantity as compared to adulterant fats have been used as an index for the detection of foreign fats in milk fat. Unusual fatty acids like iso-oleic acid, iso-valeric acid, erucic acid, cyclopropenoic acid, etc. are altogether absent in milk fat, but are found in vegetable fats.

Butyric acid is found only in milk fat, but not in adulterant fats. Some workers (Harper and Armstrong, 1954) reported that any decrease in butyric acid content of milk fat below 9.6 mole percent would indicate its adulteration with foreign fat. The presence of iso-valeric acid in dolphin oil has been used as the basis for its detection in milk fat by several workers (Tappi and Menziani, 1952; Antoniani and Cerutti, 1954) using ascending paper chromatography.

### 2.1.4.2.1 Tests for cottonseed oils

Fatty acids containing cyclopropene ring, viz., malvalic ($C_{18:-0}$) and sterculic ($C_{19:0}$) acids which are altogether absent in milk fat, but are characteristic of cottonseed oil (Pandey and Suri, 1982) have been used as a tool by few workers (Shenstone and Vickery, 1961; Christie, 1970) for the detection of cottonseed oil in milk fat and also to distinguish cotton tract ghee from normal ghee using Halphen test or methylene blue reduction test.

### 2.1.4.2.2 Halphen Test

This test is based on the development of a crimson colour due to the reaction between cyclopropenoic acids (constituents of cottonseed oil) and Halphen reagents (1% sulphur solution in $CS_2$ and equal volume of iso-amyl alcohol) after incubation for an hour in a boiling bath of saturated sodium chloride solution. This test finds its application for differentiating the cotton tract ghee from normal ghee (Singhal, 1980) as well as for the detection of cottonseed oil in milk fat.

### 2.1.4.2.3 Methylene Blue Reduction Test

Singhal (1980) developed a methylene blue reduction test for the identification of cotton tract ghee and reported that the colour of methylene blue dissolved in chloroform: methanol (1:1) was decolourised by cotton tract ghee or ghee added with cottonseed oil due to the presence of cyclopropenoic acids, while normal pure ghee did not reduce the colour of methylene blue dye.

### 2.1.4.2.4 Hydroxamic Acid test

It is a colorimetric test which is used to distinguish between butterfat and other vegetable and animal fats (Nelson, 1954) based on the fact that fats derived from milk (cow, goat, sheep, etc.) will form water soluble hydroxamic acid–iron complexes. These complexes appear as a pink to purple colour in water layer. The hydroxamic acid-iron complexes formed from fatty acid esters in vegetable fats except coconut oil and of animal fats are insoluble in water and do not contribute a distinctive pink to purple colour to the water layer. Shipe (1955 a & b) modified the above test by separating the water soluble and water insoluble hydroxamates. The soluble fraction containing the complexes of butyric, caproic, caprylic and capric acids is extracted with a butanol-ethanol mixture which removes the caprylic and capric acids and leaves the other two in the water. The relative proportion of these two pairs of acids estimated by comparing the colour intensity in the aqueous phase before and after extraction could be used to distinguish butterfat from other fats.

#### 2.1.4.2.5 Modified Bieber's Test

The Bieber's test, hitherto employed for the detection of almond oil adulteration with Kernel oil, was suitably modified by Sharma *et al.* (2007) to detect the adulteration of ghee with vegetable oils. Their results showed the presence of orange brown colour in case of refined vegetable oils & fats, whereas in case of pure ghee samples no colour was observed. By this method, adulteration of ghee with different vegetable oils to the tune of 5-7% could be detected.

The above review reveals that the detection of foreign fats in milk fat is a very complex phenomenon, almost comparable with the detection of Pacific water in a sample of Atlantic water. Although several methods based on the physico-chemical characteristics of oils and fats have been developed to detect the various types of adulterant fats such as animal body fats and vegetable oils in milk fat, but most of the methods are quite tedious, time consuming and have one or the other limitation. The detection methods available till date are mainly based on the physico-chemical constants, fatty acid profile, sterol analysis, partial solidification behaviour, etc. However, most of these methods fail when a mixture of body fats and vegetable oils & fats is added to milk fat. Therefore, the search for new and innovative method to ascertain the purity of ghee is still continued.

## 2.2 DNA based methodology for quality control in food products

Recently, DNA based methodologies have proven their potential in ensuring the quality and origin of food and feed ingredients. Literature suggested that DNA is more thermo stable than many proteins and nucleic acid is less liable to be disrupted by processing of foodstuffs (Cunningham and Meghen, 2001). Furthermore, DNA is present in the majority of the cells of an organism, potentially enabling identical information to be obtained from any appropriate sample from the same source, regardless of the tissue of origin. Additionally, through the acquisition of sequence data, DNA can potentially provide more information than protein, due to the degeneracy of the genetic code and the presence of many non-coding regions (Frezza *et al*., 2008). Among various DNA based methods PCR based methods are getting top priority in quality control analysis (Saderi *et al*., 2013).

Polymerase chain reaction (PCR) is an in-vitro enzymatic method that allows several million fold amplification of a specific DNA sequence within few hours. This technique was invented by Mullis *et al*. (1986) and is useful for food technologists. PCR makes it possible to amplify defined DNA-fragments in a very short time by a factor up to some millions in three steps (denaturation to obtain a single-stranded DNA, annealing in which primers flank the molecular region of interest, and finally extension where the DNA polymerase synthesizes the new strand). Subsequently, the amplified DNA can be analyzed by various molecular biological procedures, mainly by size fragment length polymorphism and visualized with Ethidium Bromide (EtBr) after electrophoresis in agarose gel. The decisive advance of PCR-analysis in comparison to protein chemical methods is that, in contrast to specific proteins, the whole DNA is always identically present in every organ of a species.

Nowadays, PCR assay is gaining immense popularity in identification of species origin of meat and meat products, dairy products , fish products, oil as well as oil seeds (Jain *et al*.,2007) etc. At the same time PCR is a primary part of many modern DNA based technologies. In present review the application of PCR/DNA based methodologies in meat, oil and dairy products have been discussed.

### 2.2.1 Application of PCR based methods to Authenticate meat and meat products

The identification of species origin of meat and meat products by species-specific PCR assay is simple, sensitive, and rapid as compared to other PCR assays. However, the number of species to be identified in a single PCR reaction is limited.

PCR assay is employed for the identification of species origin of meat by targeting genomic and mitochondrial DNA. This assay is very powerful for detection of species in mixed meat products (Meyer *et al*., 1994, Cunningham and Meghen, 2001 & Jain *et al*., 2007). Species-specific PCR assay has been used for species identification in meat and meat products by targeting nuclear DNA by various workers. The pork was identified in fresh and heated beef mixture by amplifying porcine-specific growth hormone gene fragment (Meyer *et al*., 1994). The mitochondrial DNA assay was applied successfully for the detection and discrimination of chicken, turkey, pig, cow and sheep tissues in animal food ingredients, using primers designed on cytochrome b gene by Herman (2001). Beef in meat products was detected using primers designed on mitochondrial cytochrome b gene by Piknova and Kuchta (2002). Swine-specific DNA primers were successfully designed for detection of pork in wide range of meat and meat products in raw and cooked meats, sausages, cured meat products, hamburgers, and patties

(Calvo *et al*., 2002b). This assay was also used for detection of pork in raw and cooked beef and duck pate by Calvo *et al*. (2002a). It was suggested that specific PCR amplification of a repetitive DNA element is a powerful technique for the identification of beef in processed and unprocessed food, because of its simplicity, specificity, and sensitivity (Calvo *et al*., 2002b). Walker *et al*. (2004) designed and evaluated species-specific PCR assays for the identification of equine, canine, feline, rat, hamster, guinea pig, and rabbit, using amplification of genome specific short and long primers. Arslan *et al*. (2006) reported that meat species could be efficiently identified using PCR assay during different cooking methods and they also reported that they found any quality degradation of DNA during various cooking methods.

The species-specific PCR identification yielded excellent results for identification of pork derivatives in food products, and it is a potentially reliable and suitable technique in routine food analysis for halal certification (Che Man *et al*., 2007). Ilhak and Ali (2007) reported that PCR can be a useful technique for fast, easy and reliable control of adulterated consumer meat products. Horse, dog, cat, bovine, sheep, porcine and goat processed meat was determined by the polymerase chain reaction (PCR) technique, using species-specific primers (Ilhak and Ali 2007). Jain *et al*. (2007) also reported that on basis of the variable region of mitochondrial gene of different species multiplex PCR technology using species specific primers were very useful for detection of meat adulteration. Frezza *et al*. (2008) successfully designed and evaluated species-specific PCR for the detection of bovine, ovine, swine, and chicken mitochondrial DNA from heat-treated material.

Mane *et al.* (2009) developed a chicken specific PCR assay for specific detection of chicken meat in mixed meat, these authors designed primer pair targeting mitochondrial d-loop resulting 442 bp amplicon from fresh, processed, and autoclaved meat and meat products. They reported that no adverse effects of cooking and autoclaving were found on amplification of chicken DNA fragments. Thus, the detection limits was found to be less than 1% in admixed meat and meat products. The developed assay was found specific and sensitive for rapid identification of admixed chicken meat and meat products processed under different manufacturing conditions.

A method based on Polymerase Chain Reaction (PCR) - Restriction Fragment Length Polymorphism (RFLP) analyses to determine the identities of meats species in meat products was assessed by Wong *et al*. (2010). Raw meat (control) and processed meat samples were analysed by targeting their cytochrome b gene. Universal primers, cytb1 and cytb2 amplified a fragment of the cytochrome b gene of approximately 360bp.Using species specific primer they were able to detect chicken meat from admixture of various type of meat.

Mane *et al*. (2012) developed a species specific PCR base technology using self-designed beef specific primer sequence for detection of beef contamination in processed meat and they reported that amplification pattern of DNA remain unchanged at high heat treatment. They found 1% beef contamination in processed meat could be detected by that method.

A specific polymerase chain reaction (PCR) method was applied by Saderi *et al*. (2013) for identification of bovine (*Bos taurus*), ovine (*Ovis aries*) and caprine (*Capra hircus*) pure and binary mixtures of raw and heat-processed meats. These meats were used in food industry as raw products and/or for direct consumption of consumers. The mitochondrial DNA was amplified using species specific primers resulted different size amplicon for different species and the authors reported that this technique was fast and reliable for identification of animal species in the mixture of meat or meat products.

### 2.2.2 Application of DNA or PCR Based Methods to Authenticate Vegetable oil

The rising interest towards the use of DNA for food authentication is also getting popularity in the case of vegetable oils. To overcome the problems of minute amounts of DNA as well as degraded DNA fragments present in vegetable oils, the amplification of small DNA fragments has been a recommended practice (Hellebrand *et al*., 1998). The 5S spacer (ribosomal DNA with internal transcribed at 5 prime end) of the DNA was successfully used to detect the presence of DNA in refined vegetable oils by multiplex PCR and real-time PCR with SYBR Green coupled to melting curve analysis. Pure sunflower and maize oils produced fragments of 108 bp and 75 bp size, respectively, reinforcing the specificity and sensitivity of the DNA analysis and suggesting its usefulness for the identification of vegetable oil adulteration (Doveri and Lee, 2007). A study has reported the successful application of a real-time PCR assay to distinguish olive oil from other plant oils, wherein by the use of olive-specific primers targeting the cDNA (complementary DNA) encoding the Olea europaea plasma intrinsic protein and a novel fluorescent dye (Eva Green) was used for real-time PCR (Wu *et al*., 2008).

To identify several vegetable oils blended in olive oil, a PCR assay coupled to capillary electrophoresis and single-strand conformation polymorphism (CE-SSCP) method targeting the rbcL gene of chloroplast genome was proposed (Wu *et al*., 2008). The method presented an absolute LOD of 0.1 pg/mL of olive DNA, a relative LOD of less than 10% DNA from other plant oils and a practical LOD of 30-50% soybean oil. Applicability to commercial oils was demonstrated, but reported that further improvements were required to increase sensitivity to vegetable oils for its effective use in olive oil authentication.

Palm oil is readily available and sold at low prices, adulteration of higher valued oils such as peanut oil and soybean oil with palm oil is currently a widespread practice (Zhang *et al*., 2009). Based on the MT3-B sequence (Melatonin receptor), conventional and real-time PCR assays were established to detect palm oil contamination by amplifying an amplicon of 109 bp. The method described by Zhang *et al*. (2009) were able to detect five haploid copies of palm DNA (10 pg) and that method was effectively applied to commercial edible vegetable oils for identification of palm oil in commercial vegetable oil.

Gimenez *et al*. (2010) determined the relative quantity of target molecules by real-time PCR using the universal SYBR Green dye of nuclear and chloroplast DNA. They reported that smaller size DNA fragments (80bp) more amplifiable than higher size (200bp) DNA fragments hence, these authors suggested that smaller size DNA fragments were much more successful to authenticate oil.

According to Spaniolas *et al*. (2010), the use of DNA maker to authenticate plant oils requires polymorphic and high copy analytical targets such as the plastid region of the Chloroplast DNA (trnL UAA) intron that has been used for discriminating several plant species.

Bai *et al*. (2011) developed a thin-film biosensor chip based analytical device to rapidly authenticate eight vegetable oils. The method used primers and probes to specifically detect canola, cotton, maize, soybean, peanut, sunflower, sesame and palm, relying on the hybridization of biotinylated PCR fragments with covalently attached probes to a thin-film silicon biosensor chip in a specific array.

Testolin and Lain (2005) and Costa *et al*. (2012) reported that the use of DNA based technology to authenticate the purity of oil is very much dependent upon the quantity of oil and size of primer.

### 2.2.3 Application of DNA based methods to authenticate milk and dairy products

The origin of dairy products from certain animal species can also be determined by DNA studies. Milk from healthy mammary glands contains a large number of somatic cells (epithelial cells and leukocytes). These cells are concentrated during processes, such as cheese making, and can be used to isolate and amplify DNA and discriminate species. In comparison with protein, DNA assays may have the advantage of increased sensitivity and rapid performance with high sample numbers being automatically processed. Furthermore, these methods could be advantageous for highly processed milk products treated with high temperature and pressure or submitted to long periods of storage, because they are methods based on more resistant material such as nucleic acids.  Lipkin *et al*. (1993) showed that it was possible to use milk as a source of DNA and as a substrate for PCR, at that time the quality of purified DNA was an unresolved problem, and some difficulties emerged in recognizing the milks of closely related species, (ewe, goat, cow, and buffalo) because of the possibility of cross-reaction. It was also not possible to define highly specific regions in genes, which could be used for an unequivocal determination by means of PCR. Furthermore, milk would seem to be a very complex food system with an abundance of potential PCR inhibitors. It has been reported (Rossen *et al*., 1992 & Plath *et al*., 1997) that certain compounds in fungi-containing cheeses (Danish blue, vein Brie, and Brie) may interact with the DNA or the enzymatic PCR reaction and inhibit it. Since then, new developments have improved the efficiency of these techniques. Such advances include rapid and reliable procedures for isolating genomic DNA from dairy samples based on the use of resins (Branciari *et al*., 2000) or spin columns (Maudet *et al.,* 2001). The finding (Poms *et al*., 2001) that free DNA is principally located in the cream fraction could be of great relevance when very small amounts of target DNA are present. Concerning the inhibitors of PCR reaction, it has been tested that the heat treatment of milk and the drying or freezing of the cheese or fungi used during cheese making did not seem to interfere with the detection method (Maudet *et al*., 2001).Specific β-casein gene PCR with universal primers encoding a partial sequence of gene was performed to detect the corresponding DNA in milk & dairy products (Plath *et al*., 1997).

### 2.2.3.1 Application of DNA based methods to authenticate milk

Mafra *et al.* (2004) have developed a duplex PCR approach to quantify the addition of cows' milk to sheep milk, which used a normalized calibration curve to control the problems associated with DNA extraction and gel preparation. Maskova and Paulíkova (2006) have used PCR method for detecting cow's milk in goat and sheep milk. Mafra *et al*. (2007) identified cow milk in sheep and goat milk by using primers targeting the mitochondrial 12S rRNA gene. The technique allowed the detection of 0.1 % of cow milk in goat milk. Zelenakova *et al.* (2009) have used PCR method for the analysis of 70 milk samples and reported that PCR based approach was very much useful to authenticate the origin of milk. Helena *et al.* (2010) used real time PCR to detect the presence of cow milk in goat milk and said authors reported that real time PCR was one of the powerful method to authenticate origin of milk Dabrowska *et al.* (2010) used cow specific primer followed by real time PCR to detect cow milk in goat milk and these authors reported that processing condition did not play any adverse effect on real time PCR assay.

### 2.2.3.2 Application of DNA based methods to authenticate Cheese

Branciari *et al*. (2000) described a procedure to determine the species of origin of milk used in cheese manufacturing, based on isolating the mitochondrial DNA and the use of PCR-restriction fragment length polymorphism of cytochrome b gene sequences. Current knowledge suggests that mitochondrial DNA may be more suitable than nuclear DNA for this kind of analysis. This material is attractive because of its variability (mitochondrial cytochrome b gene sequences differ by at least a few nucleotides, even in very closely related species), the availability of mitochondrial sequence data from many vertebrates and the high copy number relative to the nuclear DNA. In contrast, design of species-specific primers has meant that it has been possible to simplify the technique avoiding the need for subsequent restriction fragment analysis. Different works have been reported in this direction another alternative approach includes duplex-PCR (Calvo *et al*., 2001). It allows co-amplification of separate regions of a single gene or specific fragments, each typical of a different animal species in a single PCR reaction. The duplex-PCR technique has been proposed by (Bottero *et al.*, 2002) to identify bovine and water buffalo DNA in a single PCR assay in milk and mozzarella cheese originally made from pure buffalo milk. Primers were designed within the cytochrome b gene region of the mitochondrial DNA. The results of these experiments indicated the applicability of this method, which showed an absolute specificity for the two species and a high sensitivity even to low DNA concentrations. Maudet and Taberlet (2002) developed a quick and easy DNA-based test to specifically detect Holstein’s breed milk in French PDO cheeses that are not allowed to contain milk from this breed. They found a molecular marker for Holstein's milk detection in a gene affecting coat color in cattle. A single nucleotide substitution in that gene allowed the differentiation of breeds. DNA extraction from cheese, a pre-amplification of the gene and a competitive oligonucleotide priming PCR were performed.

De *et al*. (2011) developed a polymerase chain reaction (PCR), amplifying a fragment of the mitochondrial DNA d loop region for species specific detection of cattle and buffalo milk. The method was simultaneously extended for detection of HTST pasteurized milk samples and cheeses of bovine and buffalo origin. These authors used a common forward primer, with two different species specific reverse primers that resulted amplification of a 126 bp and 226 bp products for cattle and buffalo, respectively.

Recently , different researchers are recommended PCR based or DNA based technologies are always superior than any protein based methods to authenticate milk or dairy products ( Bottero *et a*l., 2002 ; Zhang *et al*., 2009; De *et al*., 2011; Feligini *et al*., 2005 and Hassan and Ali, 2012).

Although the results of PCR applied to detect the authenticity of dairy products seem reliable, some parameters are still difficult to control. The amount of DNA recoverable from milk products is directly related to the somatic cell content of the raw milk, and the somatic cell count varies according to individual modifications, diseases, and number and stage of lactation. Despite the disadvantages that still exist, the PCR-method for food analysis will become increasingly important in the future, together with fluorescence techniques to allow the quantitative determination of the sequence of DNA that is amplified, thereby avoiding analysis by gel electrophoresis by using Real Time PCR, qPCR (quantitive) PCR, PCR-HRM (curve analysis) (Feligini *et al*., 2005).

### 2.2.3.3 Application of DNA based methods to authenticate Ghee

Though lots of work has been done with PCR based methods to authenticate the milk or dairy products but very less work have been done to authenticate the milk fat. Nooratiny *et al*. (2013) developed a technique to isolate DNA from ghee using Epicentre Master DNA isolation kit followed by polymerase chain reaction (PCR) using cattle species-specific oligonucleotide primers which targeted the mitochondria DNA (mtDNA) of cytochrome b (cyt b) gene yielding 274 bp in size amplicon fragment for cattle . Hence, these authors reported that reported protocol was very efficient to prove the authenticity of ghee.

## 2.3 Tests Based on Gas Liquid Chromatography of Triglycerides (TG)

Butterfat is composed predominantly of triglycerides with 26 to 52 carbon number, while animal depot fats and common vegetable oils other than coconut and palm kernel oil have triglycerides mainly 50 to 54 carbon number. Coconut and palm kernel oil contain short and medium chain length triglycerides with 30 to 52 carbon number, a range almost similar to butterfat (Parodi, 1969; Rangappa and Achaya, 1974). The composition and distribution of TG in milk exhibit many structural similarities for different animal species (Christie, 1989). Fontecha *et al*. (1998) reported that TG content of milk fat not only varies on species to species but this variation could be observed in different breeds of same species animals. Triglyceride C38, C50 and C52 were observed maximum in cow milk fat (Smiddy *et al.* 2012). They also reported that horse milk fat contained a greater percentage of C44 whereas donkey milk had a greater percentage of C52.

Using GLC, Kuksis and McCarthy (1964) detected the presence of vegetable fat and lard in butterfat at 5 to 10 percent level based on the increase in the content of high molecular weight triglycerides, i.e. $C_{52:0}$ and $C_{54:0}$ peaks, respectively. Parodi and Dunstan (1971) reported that the trisaturated glycerides ($GS_3$) analysis could be used for the detection of beef tallow in Australian butterfat at 5 to 10 percent level. Parodi (1973) carried out GLC analysis of butterfat adulterated at various levels with beef tallow that adulteration of butterfat samples with beef tallow (20% level) and vegetable oils (5% level) was detectable. Guyot (1978) using GLC of commercial butter found that triglycerides were in the order of $C_{36:0} > C_{38:0} > C_{40:0} > C_{42:0} > C_{44:0} > C_{50:0} > C_{52:0}$. Beef tallow and lard triglycerides ranged mainly from $C_{44:0}$ to $C_{54:0}$, with $C_{52:0}$ as main triglyceride. He found the ratio of $C_{52:0}/C_{50:0}$ was less than 1 in pure butter and between 2 & 3-4, in case of beef tallow & lard, respectively. He concluded that the $C_{52:0}/C_{50:0}$ ratio together with $C_{52:0}/C_{38:0}$ ratio gave a valuable indication of the possible addition of tallow or lard to butter. Marjanovic *et al*. (1984) reported that adulteration of the milk fat with margarine at 5 to 10% level could be detected on the basis that margarine had more triglycerides with 48 to 54 acyl carbon atoms than milk fat.

Similarly, Luf *et al*. (1987) could also detect 5 to 10 percent of beef tallow and lard as well as vegetable oils / fats in butter based on $C_{52:0}$ / $C_{40:0}$ and $C_{50:0}$ - $C_{54:0}$ / $C_{38:0}$ – $C_{40:0}$ triglycerides, respectively.

Another group of researchers (Precht, 1990 & 1992) compared the triacylglycerol composition of different fats as analysed by GLC and designed a multiple linear regression equation by which foreign fats could be detected with substantially improved sensitivity. However, the method was suitable only when a single foreign fat was added to milk fat. European Union (EU) applies the method of Precht (1992) for triglyceride analysis as an official method for evaluating the milk fat purity. Povolo *et al*. (1999) applied the above said official method of EU coupled with the determination of 3,5-cholestadiene content (Mariani *et al.,* 1994) and reported that the detection of beef tallow up to 0.5 to 1.0 percent using 3,5-cholestadiene analysis and up to 2 percent using multivariate statistical techniques could be done. Several investigators (Contarini *et al*., 1993; Renterghem, 1997; Collomb, 1997; Banfi *et al.*, 1999) carried out the triacylglycerol analysis by GLC and applied it to detect adulteration of milk fat. Pinto *et al.* (2002) did linear discriminant analysis of triacylglycerides to determine the authenticity of pure milk fat. The objective of the study was to propose a methodology for the detection of adulteration of milk fat. Naviglio and Raja (2003) proposed a gas chromatographic analysis of butter using a capillary column having 65 percent phenyl methyl silicone as stationary phase. This method allows the detection of extraneous vegetable and animal fats in a simple, rapid and precise way, even at lower levels.

Kala (2013) reported that 18 types of triglyceride (TG) found in ghee but C36 to C54 TG were predominant where as in case of lard C50, C52 and C54 were main TG but TG C24-C32 were absent in lard. The same author also reported that main TG for partially hydrogenated vegetable oil were C34, C36, C48, C50, C52 and C54 but in case of coconut oil C34,C36, C38, C40, C42 and C44 were the predominant TG. Same type of observations were reported by Kim *et al.* (2015), who reported that all 18 TGs (C24 to C54 only even number) were present in milk fat but in case of lard C50-C54 were the predominant. They also reported that cow milk fat adulterated with lard showed an increase TG 52 and 54. In another study carried out by Destaillats *et al*. (2006), who reported that adulteration of milk fat with partially hydrogenated vegetable oil with milk fat showed an increase in TG 36 and 54.

Rakesh (2016) reported that adulteration of coconut oil alone and in conjunction with lard in buffalo ghee could be detected at 5% level by observing the increase in sum of Triglycerides carbon number (C34+C36+C38), also adulteration of buffalo tallow, goat tallow and lard in both type of ghee (cow and buffalo) could be detected at 5% level by observing the increase in sum of Triglycerides (C50+52+54).

European Union has selected TG analysis using packed or capillary column and low-resolution gas chromatography (GC) methodology as official reference method to detect non-milk fat in milk fat. International Organization for Standardization (ISO) and International Dairy Federation (IDF) jointly specify a method to detect non-milk fat adulterants in milk fats based on TG compositions and in combination with standardised formula known as standardised (S) values (S Total, S2, S3, S4 and S5) for different kinds of adulterants (IDF & ISO 2010).Details of equations are given below-

1- Stotal (95.68–104.32) (General formula) -2.7575C26 + 6.4077C28 + 5.5437C3015.3247C32 + 6.2600C34 + 8.0108C40-5.0336C42 + 0.6356C44 + 6.01171C46.

2- S2 (98.05–101.95) (Soya bean, Sunflower, olive, Rape-seed, Linseed, Wheat germ, maize germ, Cotton seed & Fish oil) = 2.0983C30 + 0.7288C34 + 0.6927C36 + 0.6353C38 + 3.7452C40-1.2929C42 + 1.3544C44 + 1.7013C46 + 2.5283C50.

3- S3 (99.42–100.58) (Coconut & Palm kernel fat) = 3.7453C32 + 1.1134C36 + 1.3648C38 + 2.1544C42 + 0.4273C44 + 0.5809C46 + 1.2926C48 + 1.0306C50 + 0.9953C52 + 1.2396C54.

4- S4 (95.90–104.10) = (Palm oil & beef tallow) = 3.6644C28 + 5.2297C30-12.5073C32 + 4.4285C34-0.2010C36 + 1.2791C38 + 6.7433C40_4.2714C42 + 6.3739C46

5- S5 (97.96-102.04) (Lard) = 6.5125C26 + 1.2052C32 + 1.7336C34 + 1.7557C36 + 2.2325C42 + 2.8006C46+ 2.5432C52 + 0.9892C54

There were limitations indicated for the IDF & ISO (2010) reference method that this method is not applicable to the milk fats other than cow's milk, the milk obtained was not from a single cow, obtained from skim milk or butter milk, the extracted fat should be exclusively from gravimetrical methods, and the milk obtained by the animal receiving high feeding of pure vegetable oils (Kala, 2013).

In Indian context IDF & ISO (2010) equation is not extensively studied for ghee as in India, feeding pattern of animals varies region wise, more over there is a great diversification of breed and species from one region to another region, in this case IDF&ISO (2010) equation should be validated and studied extensively in Indian scenario.

## CHAPTER - 3

# Materials and Methods

# 3. MATERIALS AND METHODS

The chapter deals with the research design and methodological procedure used for the present investigation to achive the foregoing objectives. The material used and method adopted in the present investigation are discussed below:

## 3.1 Chemicals, Reagents and Equipments

**3.1.1 Chemicals:** EDTA-Ethylenediaminetetraacetic acid, NaCl-Sodium chloride , SDS-Sodium dodeycyl sulfate, Sodium Acetate(3M, pH 5.3), (Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India).Proteinase-K, Agarose powder, Taq-Polymerase(5U-5 unit),RNAse free water, dNTPS (deoxynucleotide phosphate), ethidium bromide (Sigma Aldrich,USA),Molecular marker -100bp 50bp gene ruler & 6X Loading dye (Fermentas, Germany) ,Commercial lard (Biomedical, USA).

**3.1.2 Reagents:** DNA extraction buffer (75 mM NaCl, 2 mM EDTA, 10 mM Tris-Cl pH 8.0), Tris saturated phenol (pH 8.0) , CTAB-*cetyltrimethylammonium bromide* (2% CTAB, 0.8 M NaCl, 50 mM Tris-HCl, 1 mM EDTA), CTAB precipitation solution (5 g/L CTAB, 0.04 mol/L NaCl), TENS buffer-Tris EDTA, Sodium Hydroxide ,SDS (l0mM Tris, pH 8.0, 1mM EDTA; l00mM Nacl and 0.5% SDS) ,TE-(Tris EDTA)-buffer (Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India) ,Phosphate buffer solution (PBS- pH 7.4) (Sigma Aldrich,USA).

**3.1.3 Solvents:** Phenol, chloroform and iso-amyl alcohol (AR grade, S.D. Fine-chem Ltd., Mumbai, India), Ethanol absolute, Hexane (S.D. Fine-chem Ltd., Mumbai, India), Isoamyl alcohol molecular grade, Molecular Grade Tris saturated phenol (Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India).

**3.1.4 Reference standards:** Triglyceride (TG) mixture (C24, C30, C36, C42, C48) (Sigma- Aldrich Chemie,USA).

**3.1.5 DNA isolation kits:** Nucleo Spin Tissue kit mini (MACHEREY- NAGEL, Germany), QIAamp DNA stool and DNeasy mericon food kit (QIAGEN,Germany)

**3.1.6 Primers:** All oligonucleotide Primers got synthesized from (Sigma,USA)

**3.1.7 Equipments:**

**3.1.7.1 Boiling water-bath (rectangular):** Perfit India Pvt. Ltd. Ambala, India

**3.1.7.2 Boiling water-bath (circular):** Tempo Industrial Corporation, Bombay.

**3.1.7.3 BOD Incubator:** Metrex Scientific Instruments Pvt. Ltd., New Delhi.

**3.1.7.4 Cream separator:** Kamdhenu, KD-60E, Benny Impex, New Delhi, India.

**3.1.7.5 Digital temperature control oven:** Narang Scientific Works Pvt. Limited, New Delhi, India.

**3.1.7.6 Digital multipurpose thermometer:** Hangzhou Huaan Medical & Health Instruments Co., Ltd., China.

**3.1.7.7 Electric heater:** Vikrant, Jain Enterprises,Gulabi Bagh,Delhi, India.

**3.1.7.8 Electronic balance:** Citizen Scale India Pvt. Ltd., India.

**3.1.7.9 Gas liquid chromatograph:** Model: GC 2010 PLUS, SHIAMDZU, Japan.

**3.1.7.10 Refrigerator:** LG Electronics India, India.

**3.1.7.11 Nano Drop spectrophotometer-** TM IMPLEN (GmbH, Munich, Germany)

**3.1.7.12 PCR**: Auto cycler, Bio Rad, USA**.**

**3.1.7.13 Electro phoresis unit**: L-300, Genetix Bio,India

**3.1.7.14 Gel Doc unit**: e-Gel Document, Thermo Fisher, USA

**3.1.7.15 Trans illuminator system**: Thermo Fisher, USA

**3.1.7.16 Table top mini centrifuge**: Genetix, Germany.

**3.1.7.17 Table top clinical centrifuge**: Remi, India.

**3.1.7.18 Vacuum concentrator:** Ecospin 3180-C,Hanil scientific, Industry, Korea.

### 3.1.8 Laboratory consumable items:

**3.1.8.1 Filter paper:** Whatman filter paper No.4, England.

**3.1.8.2 Nitrile Gloves**, Tarson Products Pvt. Ltd., India.

## 3.2 Collection and Preparation of Samples

### 3.2.1 Collection of Milk and Preparation of Ghee

Cow and buffalo milk, used for the preparation of respective ghee samples, were collected after every two consecutive months up to complete one year (November, January, March, May, July and September) from the Livestock Research Centre, NDRI, Karnal. Cow milk was bulk milk consisting of mixture of the milk obtained from the herd of Karan Swiss, Karan Fries, Sahiwal and Tharparkar breeds. Buffalo milk used was also bulk milk obtained from Murrah breed only. Cows and buffaloes were maintained under identical conditions of feeding and management. The fodder/roughage given to animals throughout the year consisted of berseem, mustard, oat, turnip, maize and silage (jowar and maize) in November to April while maize, jowar, cowpea and lusan in May to October. In July and August silage of maize, jowar and oats while from November to March oat hey was also given to lactating cattle. The concentrate mixture comprised of mainly maize (>30%), barley, wheat or oat, groundnut cake (22%), mustard cake (11%), wheat bran (25%), rice bran de-oiled (8%), minerals mixture (2%) and common salt (1%).

Samples of cow/buffalo ghee were prepared by creamery butter method (De, 2005). Soon after the collection of milk samples, these were warmed to 40°C and separated into cream using mechanical cream separator. The cream was pasteurized at 77$^{o}$C for 5 minutes, cooled to room temperature and then kept in a refrigerator (5 to 10$^{o}$C) for few hours (3 to 5 hours) for aging. Butter was prepared under standard conditions (9°C in summer and 13°C in winter) by churning of 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 cloth followed by further filtration by using Whatman No.4 filter paper in glass vacuum assembly and finally filled in plastic bottles, cooled to room temperature and kept in a refrigerator at a temperature of 5 to 10°C till further analysis. The samples of cow and buffalo ghee thus prepared each after every two consecutive months for complete one year were used as control samples as well as for the preparation of adulterated ghee samples.

The flow chart for the preparation of control ghee samples was as follows:

**Collection of cow/buffalo milk**
↓
**Cream separation (at 40$^{o}$C, Ageing temp 4-5 $^{o}$C)**
↓
**Churning to butter**
↓
**Clarification at 120$^{o}$C/ flash**
↓
**Filtration (Muslin cloth and W.No. 4 filter paper)**
↓
**Ghee**

### 3.2.2 Procurement of market sample of Ghee and cotton tract area Ghee

Market sample of Ghee were procured from local market of Karnal and homemade buffalo ghee samples were procured from cotton tract area of Gujarat, Amreli.

### 3.2.3 Procurement and preparation of adulterant oil and fat

Vegetable oil (Soyabean oil –Soy oil) and body fat (Pig and Goat body fat ) were used as adulterant oil/fat in the present investigation.

#### 3.2.3.1 Heat clarified animal body fat (HCABF)

Goat adipose tissue was collected from local Bakra market, Sadar Bazar, Karnal. Pig adipose tissue was collected from local slaughter house situated in Hanshi road, area of Karnal. Adipose tissues were first washed thoroughly under running tap water and then heated at about 140°C over a direct flame till a transparent liquefied body fat was obtained. The liquid fat was filtered through muslin cloth followed by Whatman filter paper No. 4 before its final use.

#### 3.2.3.2 Vegetable oil

Soyabean oil (Soy oil) was purchased from local market of Karnal.

### 3.2.4 Blood sample collection

Goat blood was collected from local Bakra market, Sadar Bazar,Karnal. Pig blood was collected from local slaughter house situated in Hanshi road, area of Karnal.

## 3.3 Preparation of Adulterated Ghee Samples

For the preparation of adulterated ghee samples, pure ghee samples (**3.2.1** ) were heated to 60-70°C for 10 min followed by the addition and mixing of molten adulterants.

## 3.4 Isolation of DNA from Ghee and heat clarified animal body fat

DNA from ghee and heat clarified animal body fat was isolated by the following modified method. Quality and quantity of isolated DNA from fat samples was established by running the DNA sample on 0.8% agarose gel electrophoresis as well as by measuring absorbance on nano drop spectrophotometer.Also universal primers (1,2) were used to check the amplification quality of DNA.

### 3.4.1 Modified QIAamp stool kit based Method for isolation of DNA from Ghee and heat clarified animal body fat

In this case, a method as described by Kumar *et al.* (2011a) for DNA isolation from olive oil was used with some modifications and the method is as follows

Forty five militre (45ml) of fat sample was divided equally in,3 centrifuge tubes of 50 ml capacity.Then equal volume of ASL buffer (15 ml) was added in each of the three tubes and vortexed for 1 min followed by incubation at 75º C for 5 min. Mixture was re-vortexed for 15 s, followed by spinning for 5 min at 8000 rpm. The lower clear phase was transferred to a new 50 ml centrifuge tube. Three to four ‘‘Inhibit EX Tablets’’ were added to the lower phase collected from previous step, vortexed immediately for 1 min and incubated at room temperature for 1 min. Tube was centrifuged for 5 min at 8000 rpm. The upper transparent solution (containing DNA) was transferred to a new 50ml centrifuge tube followed by sequential addition of 1/10 volume of 3 M Sodium Acetate (5.3 pH) and 1 volume of absolute ethanol. The mixture was kept in -20ºC for 45-60 min, spinned for 2 min at 8000 rpm and thereafter supernatant (SN) was discarded. Pellet or suspended material was re-suspended into 400 µl sterilized water and followed by addition of 30 µl proteinase-K and 400 µl AL buffer provided in the kit. The contents were incubated for 10 min at 70º C. Afterwards 800 µl absolute ethanol was added to the content of the tube and transferred to QIAamp spin column. The column was spinned for 1 min at 14,000 rpm to bind the DNA, and filtrate was discarded. The column was washed first with 500 µl AW1 buffer by spinning at 14,000 rpm for 1 min, washed with 500 µl AW2 buffer and re-spinned for 2 min at 14,000 rpm. The column was dried at 14,000 rpm for 1 min and pure DNA was eluted using 25 µl of warmed AE buffer (65ºC) and centrifugation at 14,000 rpm for 1 min.

### 3.4.2 Evaluation of the quality and quantity of isolated DNA

For evaluating the quality and quantity of DNA isolated from fat sample, 0.8% agarose gel electrophoresis and nano drop spectrophotometer analysis (reading), was respectively carried out.

#### 3.4.2.1 Assessment of the quantity of isolated DNA using Nano Drop Spectrophotometer

The quantity and purity of extracted DNA was analysed by spectrophotometry using Nanophotometer-TM IMPLEN (GmbH, Munich, Germany). The DNA concentration was determined by UV absorbance at 260 nm. The purity of the extracted DNA was determined by the ratio of the absorbance at 260 and 280 nm. As per the literature the ratio of pure DNA should be 1.8 (De *et al.,* 2011). Any deviation from this value indicates that the DNA conatins some impurities.

#### 3.4.2.2 Evaluation of the quality of isolated DNA on 0.8% agarose gel electrophoresis

Twelve micro liter (12 μl) of isolated DNA was mixed with 2 μl of 6X gel loading dye and electrophoresed on 0.8% agarose gel containing ethidium bromide (at the rate of 0.5 μg/ml of gel solution) at constant voltage i.e 30V for 60 min in 1 X TE (Tris EDTA) buffer. The product was visualized under UV light and documented by gel documentation system.

### 3.5 Evaluation of the quality of isolated DNA by Polymerase Chain Reaction (PCR) using universal primer (1&2)

The Polymerase chain reaction (PCR) is an *in vitro* enzymatic method that allows several million fold amplification of a specific DNA sequence within few hours.

This technique was invented by Mullis *et al*. (1986) and it is also useful for food technologists.

For checking the amplification behavior of isolated DNA from ghee or fat samples, cytrochrom b (*cytb)* gene *and 12srRNA* genes were selected.Two universal primers were shortlisted from literature considering that these primers were able to amplify area of *cytb gene and 12srRNA* gene across different species.

### 3.5.1 PCR using universal primer-1

Kocher *et al.* (1989) used the following set of primer for studying the evolution of mitochondrial DNA across the animal species and the authors defined this set of primer as universal primer. Details of this primer is given below-

**Characterstics universal primer-1**

| **Targeted Gene** | **P-F(5ˡ-3ˡ) (Forward Primer)** | **P-R(5ˡ-3ˡ) (ReversePrimer)** | **Size of the amplicon** | **Reference** |
|---|---|---|---|---|
| *Cytb* | CCA TCC AAC ATC TCA GCA TGA TGA AA | GCC CCT CAG AAT GAT ATT TGT CCT CA | 360bp | Kocher *et al.* (1989) |

PCR was set up in a reaction volume of 25 µl with about 25 ng of genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 5 pmol of each forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| **PCR CONDITIONS** | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/2min | 94ºC / 5 s | 55ºC / 30 s | 72ºC / 40 s | 72ºC / 2 min |
| | 35 cycles | | | |

### 3.5.2 PCR using universal primer-2

Kocher *et al.* (1989) used this set of primer for studying the evolution of mitochondrial DNA by targeting *12srRNA* gene across the animal and the author defined this set of primer as universal primer. Details of this primer are given below-

**Characterstics of universal primer-2**

| **Targeted Gene** | **P-F(5'-3') (Forward Primer)** | **P-R(5'-3') (Reverse Primer)** | **Size of the amplicon** | **Reference** |
|---|---|---|---|---|
| *12srRNA* | CAA ACTGGG ATT AGA TAC CCC ACT AT | GAGGGT GAC GGG CGG TGT GT | 440bp | Kocher *et al.* (1989) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$, 10 pmol primer of each forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).The condition to set up PCR are as follows-

| **PCR CONDITIONS** | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 94°C / 50 s | 62°C / 50 s | 72°C / 60 s. | 72°C /10 min. |
| | 35 cycles | | | |

### 3.5.3 Gel electrophoresis to check the size of the product formed after PCR amplification

To confirm the amplification of targeted PCR product, 6 µl of the PCR product was mixed with 1 µl of 6X gel loading dye and electrophoresed along with DNA molecular weight marker (Gene Ruler, Fermentas) on 2.0% agarose gel containing ethidium bromide (0.5 µg/ml of gel solution) at constant voltage i.e 90V for 30 min in

1 X TE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

### 3.6 Isolation of DNA from blood

Blood is considered to be a good source for isolation of DNA. Thus for checking the efficacy and specificity of oligonucleotide primers, blood from various animal species like goat,cow, buffalo and pig was used to isolate DNA for downstream application. Genomic DNA was extracted from blood of cow, buffalo, pig and goat using phenol- chloroform extraction method (Sambrook and Russell, 1989) with minor modifications. The details of the method are as follows:

Ten milliliter (10 ml) blood was taken in an Oakridge tube and two volumes of ice-cold RBC (Red blood cell) lysis buffer was added. Tubes were incubated in the ice bath for 10 minutes, followed by centrifugation at 8,500 rpm for 10 minutes at $4^{o}$C. White blood cell (WBC) pellet was re-suspended in one volume of RBC lysis buffer, incubated in ice for 10 minutes, followed by centrifugation as described earlier. RBC lysis was repeated until a clear pellet of white blood cells was obtained. WBC pellet was re-suspended in DNA extraction buffer (equal to the volume of blood sample) and after adding SDS (20%) and Proteinase-K (@ 40 μl (20 mg/ml stock solution) per 10 ml of blood), incubated at $56^{o}$C for 4-6 hours. Equal volume of Tris (pH 8.0) saturated phenol was added to the above solution and mixed gently by inversion so as to form a uniform suspension (5-10 minutes). Tubes were centrifuged at 10,000 rpm for 15 minutes at $20^{o}$C. Since the DNA will partition into upper aqueous phase, it was gently aspirated (without disturbing the interphase of protein) using a wide bore sterile Pasteur pipette, and transferred to a fresh Oakridge tube. A solution of saturated phenol, chloroform and isoamyl alcohol (25:24:1) was added in equal volume followed by mixing, centrifugation and collection of aqueous phase.

The process was repeated, as described earlier. This process was further extracted with solution of chloroform and isoamyl alcohol (24:1). The aqueous phase was aspirated into a fresh sterile glass tube and DNA was precipitated by adding Sodium acetate (3 M, pH 5.3) to a final concentration of 0.33 M, and two volumes of ice-cold ethanol. The DNA was spooled out in an eppendorf tube followed by washing with 70% ethanol. After drying the DNA in a sterile incubator at 37°C, it was dissolved in 500 µl TE buffer (pH 8.0).

### 3.7 Standardization of Genomic DNA based methods for detection of body fat adulteration in ghee

This was the second objective of the study. To accomplish this objective of detection of animal body fat in ghee by using DNA based methodology, mainly **species specific PCR based approach** approaches was explored while checking the specificity and sensitivity of selected primers, following points were taken into consideration-

- ✓ Primers from literature were selected to amplify various regions of targeted gene/genes
- ✓ Specificity was considered to be amplification in only desired single species.

### 3.7.1 Sample preparation for finding detection limit in adulterated ghee using species specific primers

For estimating the detection limit of the selected primer, ghee (cow/buffalo) adultrated with body fat (tallow / lard) alone as well as in conjunction with vegetable oil (soy oil) at various level was used. Earlier researchers used (5%,10% & 20%) level of adulterants for adultration in ghee (Patel, 2011; Kumar 2013 & Upadhyay 2014 ) so we used this same ratio.The details are as follows:

| Sr. No | Samples | Adulterant | Levels of addition to ghee (%) | | |
|---|---|---|---|---|---|
| 1 | Cow Ghee | Tallow | 20 | 10 | 5 |
| 2 | Buffalo Ghee | Tallow | 20 | 10 | 5 |
| 3 | Cow Ghee | Lard | 20 | 10 | 5 |
| 4 | Buffalo Ghee | Lard | 20 | 10 | 5 |
| 5 | Cow Ghee | Lard+Soy oil | 10+10 | | |
| 6 | Buffalo Ghee | Lard+Soy oil | 10+10 | | |
| 7 | Cow Ghee | Goat Tallow+Soy oil | 10+10 | | |
| 8 | Buffalo Ghee | Goat Tallow+Soy oil | 10+10 | | |

### 3.7.2 Detection of Lard in ghee using various Pig specific Primer

### 3.7.2(A) Primer (L1)

In this case a set of primer targeting mitrochondrial d loop (mt-d-loop) of mitrochondria was selected from literature (Mane *et al*., 2013). Primer characterstics are given below-

**Characterstics of Primer (L1)**

| Targeted region | P-F($5^{I}$-$3^{I}$) (ForwardPrimer) | P-R($5^{I}$-$3^{I}$) (ReversePrimer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| mt-d loop | GCA AAC CAA AAC GCC AAG TAC T | GGT GGT GAT ATG CAT GTT GAC TG | 276bp | Mane *et al.* (2013) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 50 pmol primer (P-1), 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3). Conditions of set up PCR is given below-

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/ 2min | 94ºC / 50 s | 60ºC / 50 s | 72ºC / 60 s | 72°C /5 min |

35 cycles

**3.7.2(B) Primer (L2)**

In this case a set of primer targeting *cyt-b* gene was selected from literature (Kitpipipt *et al*., 2014). Primer specification and PCR condition is given below-

**Characterstics of Primer (L2)**

| Targeted Gene | P-F(5'-3') (Forward Primer) | P-R(5'-3') (ReversePrimer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| *Cyt-b* | GAA AAA TCA TCG TTG TAC TTC AAC TAC A | GGT CAA TGA ATG CGT TGT TGA | 100bp | Kitpipipt *et al.* (2014) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 10 pmol of each forward and reverse primer , 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3). Condition of set up PCR is as follows:

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 98ºC / 5 s | 66ºC / 5 s | 72ºC / 25 s | 72°C /1 min |

35 cycles

### 3.7.2(C) Primer (L3)

In this case a set of primer targeting mt-d-loop region of mitrochondria was selected from literature (Nagappa *et al*., 2014). Primer specification and PCR condition is given below-

**Characterstic of Primer (L3)**

| Targeted region | P-F(5'-3') (Forward Primer) | P-R(5'-3') (ReversePrimer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| mt-d loop | ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC A | CTA CGA GGT CTG TTC CGA TAT AAG G | 712bp | Nagappa *et al.* (2014) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 10 pmol of each forward and reverse primer , 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/2min | 94ºC / 30 s | 60ºC / 60 s | 72ºC / 30 s | 72°C /5 min. |
| | 32 cycles | | | |

### 3.7.2(D) Primer (L4)

In this case a set of primer targeting *cyt-b* gene was selected from literature (Matsunaga *et al*., 1999). Primer specification and PCR condition is given below-

**Characterstics of Primer (L4)**

| Targetd Gene | P-F(5'-3') (Forward Primer) | P-R(5'-3') (Reverse Primer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| *Cyt-b* | GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA | GCTGATAGTAGATTTGTGATGACCGTA | 440bp | Matsunaga *et al*.(1999) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 10 pmol forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).PCR condition as follows-

| **PCR CONDITIONS** | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 94ºC / 50 s | 60ºC / 50 s | 72ºC / 50 s | 72°C /5 min |
| | 35 cycles | | | |

**3.7.2(E) Primer (L5)**

In this case a set of primer targeting *cyt-b* gene was selected from literature (Dooley *et al*., 2004). Primer specification and PCR condition is given below-

**Characterstic of Primer (L5)**

| **Targeted Gene** | **P-F(5'-3') (Forward Primer)** | **P-R(5'-3') (ReversePrimer)** | **Size of the amplicon** | **Reference** |
|---|---|---|---|---|
| Cyt-b | ATG AAA CAT TGG AGT AGT CCT ACT ATT TAC A | CTA CGA GGT CTG TTC CGA TAT AAG G | 149bp | Dooley *et al.* (2004) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 2.5 mM $MgCl_2$, 10 pmol forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 94ºC / 30 s | 60ºC / 60 s | 72ºC / 30 s | 72°C / 5 min |
| | 35 cycles | | | |

### 3.7.2.1 Evaluation of PCR products

PCR products were checked by 2% agarose gel electrophoresis by the following way

To confirm the targeted PCR amplification, 6 µl of the PCR products from each tube was mixed with 1 µl of 6X gel loading dye and electrophoresed along with DNA molecular weight marker (Gene Ruler, MBI Fermentas, Quiagen,Germany) on 2.0% agarose gel containing ethidium bromide (at the rate of 0.5 µg/ml of gel solution) at constant 90V for 30 min in 1 X TE buffer. The amplified product was visualized as a single compact band of expected size under UV light and documented by gel documentation system.

### 3.7.3. Detection of goat tallow in ghee by using Goat specific primer

### 3.7.3 (A) Primer (T1)

In this case a set of primer targeting *cyt-b* gene was selected from literature (Matsunaga *et al*., 1999). Primer specification and PCR condition is given below-

**Characterstics of Primer (T1)**

| Targeted Gene | P-F(5'-3') (Forward Primer) | P-R(5'-3') (Reverse Primer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| *Cyt-b* | GACCTCCCAGCTCCATCAAACATCTCATCTTGATGAAA | CTCGACAAATGTGAGTTACAGAGGGA | 157bp | Matsunaga *et al*. (1999) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$10 pmol forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 94ºC / 50 s | 60ºC / 50 s | 72ºC / 50 s | 72°C / 5 min |
| | 35 cycles | | | |

**3.7.3 (B) Primer (T2)**

In this case a set of primer targeting *cyt-b* gene was selected from literature (Maria-Lopez-Andreo *et al*., 2005). Primer specification and PCR condition is given below-

**Characterstic of Primer (T2)**

| Targeted Gene | P-F(5'-3') (Forward Primer) | P-R(5'-3') (Reverse Primer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| *cyt-b* | GACCTCCCAGCTCC ATCAAACATCTCATC TTGATGAAA | AAA TAT TTG ATG GAG CTG GGA GA | 119bp | Maria-Lopez-Andreo *et al*. (2005) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$, 10 pmol forward and reverse primer , 2.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 98ºC / 5 s | 66ºC / 5 s | 72ºC / 25 s. | 72°C /1 min. |
| | 35 cycles | | | |

### 3.7.3 (C) Primer (T3)

In this case a set of primer targeting *cyt-b* gene was selected from literature (Nagappa *et al., 2011*). Primer specification and PCR condition is given below-

**Characterstic of Primer (T3)**

| Targeted Gene | P-F($5^{\prime}$-$3^{\prime}$) (Forward Primer) | P-R($5^{\prime}$-$3^{\prime}$) (Reverse Primer) | Size of the amplicon | Reference |
|---|---|---|---|---|
| *Cyt-b* | CCA CCC ACG GAC ACGAG | AGT TCA ATG CCC TAT ATGCTT CAG | 404bp | Nagappa *et al*. (2011) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$, 10 pmol forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| Initial denaturation | Denaturation | Annealing | Extension | Extension Final |
| 94°C/5min | 94ºC / 30 s | 60ºC / 30 s | 72ºC / 30 s. | 72°C /10min. |
| | 30 cycles | | | |

### 3.7.3 (D) Primer (T4)

In this case a set of primer targeting *cyt-b* gene was selected from literature (Kumar *et al., 2011b*). Primer specification and PCR condition is given below-

**Characterstic Primer (T4)**

| Targeted Gene | P-F($5^{\prime}$-$3^{\prime}$) (Forward Primer) | P-R($5^{\prime}$-$3^{\prime}$) (Reverse Primer) | Size of the amplicon (bp) | Reference |
|---|---|---|---|---|
| *Cyt-b* | TTCTTCAGGGCCATCT CATC | GCGGATGCATGGTG AAAT | 294bp | Kumar *et al*. (2011b) |

PCR was set up in a reaction volume of 25 µl with about 25 ng genomic DNA using i-cycler (BioRAD, USA). The reaction mixture consisted of 200 µM- (dNTPs) each of dATP, dCTP, dGTP, dTTP, 3.5 mM $MgCl_2$, 10 pmol forward and reverse primer, 1.0U *Taq* polymerase (Sigma-Aldrich®) and corresponding *Taq* buffer-10 mmol Tris-HCl (pH 8.3).

| PCR CONDITIONS | | | | |
|---|---|---|---|---|
| **Initial denaturation** | **Denaturation** | **Annealing** | **Extension** | **Extension Final** |
| 94°C/5min | 94ºC / 30 s | 56ºC / 30 s | 72ºC / 30 s | 72°C / 7 min |

30 cycles

### 3.8 Low- Resolution GLC analysis of Triglyceride

The third section of this thesis deals with the objective **to differentiate the pure ghee and adulterated ghee on the basis of triacylglycerol profile using GLC**. Triglyceride profiling of heat clarified fat (control and adulterated samples) was carried out by low- resolution chromatography using Shimadzu 2010 Plus GC (Kyoto, Japan) equipped with flame ionization detector and temperature control module. Nitrogen was used as carrier gas. BP-5 capillary column 2.5 m length (cut from 15 m X 0.25 mm X 0.25 µm) was used at a column flow of 1.49ml/min. One µl of 5 mg/ ml of pure cow ghee, buffalo ghee, goat tallow, lard , adulterated samples and Supelco (Cat No. 17811) TG standard mix (5 TG mix – 100 mg neat mixture of 99% C8:0, C10:0, C12:0, C14:0 and C16:0 acids) dissolved in hexane was injected in split mode at a split ratio of 1:50 for the analysis. Gas liquid chromatographic conditions (Kala, 2013) for the efficient separation of triglycerides were standardised. The details of the Gas chromatograph conditions used were as follows: injector and detector temperatures were 350°C and 370°C, respectively. Initial oven temperature was 80°C held for 0.50 min. and then raised to 190°C at 50°C/min and held for 1 min. Thereafter, it was raised to 335°C at 6°C/min and held for 6min.

**Details of temperature programmed**

| Ramp Rate | Temperature | Hold Time |
|---|---|---|
| - | 80°C | 0.50 min |
| 50°C/min | 190°C | 1min |
| 6°C/min | 335°C | 5min |

### 3.8.1 Specification for running the Gas liquid chromatograph

#### 3.8.1.1 Injector Condition

1) Temp of injector-350°C
2) Split ratio-1:50
3) Pressure-13.4Kpa
4) Total Flow 79ml/min ($N_2$)
5) Column Flow 1.49ml/min
6) Linear velocity-56.4cm/s
7) Purge flow3ml/min
8) Injection volume-1µl

#### 3.8.1.2 Column Information

For analysis of triglycerol we used BP5, capillary column 2.5 m length (cut from 15 m X 0.25 mm X 0.25 µm).

1) Name-BP5
2) Length- 2.3m, Inner Dia-.25mm,Film Thickness-.25µm
3) Max temp-335°C

#### 3.8.1.3 Detector (FID) Condition

1) FID temperature-370°C
2) Make up flow-30ml/min, $H_2$ flow -40ml/min, Air flow-400ml/min

GC solution software provided by the GLC Company was used to determine the percentage of different tri-glycerol present in different fat sample

## 3.9 Sample pattern for Triglyceride profiling for pure ghee, adulterants, and adulterants added in pure ghee

| Sr. No. | Samples (Ghee) | Adulterants | Levels (%) v/v |
|---|---|---|---|
| 1 | Pure Ghee (cow and buffalo), cotton tract area ghee ( buffalo) | Soy oil ,Goat tallow, Lard | |
| 2 | Cow ghee + Adulterant | Lard | 5, 10, 20 |
| 3. | Buffalo ghee + Adulterant | Lard | 5, 10, 20 |
| 4. | Buffalo ghee + Adulterant | Goat Tallow | 5, 10, 20 |
| 5. | Cow ghee + Adulterant | Goat Tallow | 5, 10,20 |
| 6. | Buffalo ghee + Adulterant | Soy oil | 5, 10, 20 |
| 7. | Cow ghee + Adulterants | Soy oil | 5,10,20 |
| 8. | Cow ghee + Adulterants | Soy oil+ Lard | 5+5 |
| 9. | Cow ghee + Adulterants | Soy oil+ Goat Tallow | 5+5 |
| 10. | Buffalo ghee + Adulterants | Soy oil+ Goat Tallow | 5+5 |
| 11. | Buffalo ghee + Adulterants | Soy oil+ Goat Tallow | 5+5 |

### 3.9.1 Sample Preparation

Molten and filtered fat sample was dissolved in Hexane in such proportion so that the concentration of the sample was 5 mg/ml. To get this concentration 25mg of molten fat sample was transferred to a 5ml volumetric flask and volume was made to 5ml with hexane. Contents of the flask were mixed gently for 1 min to have a uniform sample.

## 3.10 Statistical analysis

Data reported were analyzed using program of CSAT6 and expressed as mean values with standard deviation.

## CHAPTER - 4

# Results and Discussion

# 4. RESULTS AND DISSCUSSION

This chapter deals with the results obtained during the present study. The whole part has been discussed in three phases as per the three objectives. The first phase of this study deals with the optimization of a process for the isolation of DNA from pure ghee and heat clarified animal body fat (goat tallow and lard). The second phase deals with the standardization of genomic DNA based methods for detection of body fat adulteration in ghee and the third phase deals with the differentiation of pure ghee and adulterated ghee on the basis of triglyceride profile using GLC.

## 4.1 Optimization of a process for the isolation of DNA from fats [pure ghee and heat clarified animal body fat (goat tallow and lard)]

Ghee and heat clarified animal body fats consist of more than 99% of triglyceride and very small amount of moisture. Therefore, it is very difficult to isolate genomic DNA from these fats especially ghee, which is prepared after various processing steps like separation of cream from milk, preparation of butter and finally heat clarification of butter or cream to ghee. At the same time, this rigorous processing results in almost absence of any adipose tissue or other somatic cells in the ghee. Literature also suggested that in case of complex and highly processed food matrices, the critical step for application of molecular approaches is the DNA extraction and purification (Costa *et al*., 2012). Adequate strategies are required to ensure efficient recovery of minimally denatured nucleic acids and removal of PCR inhibitors. These inhibitors can interfere during various steps in the PCR, causing a decrease or even a complete inhibition of *DNA polymerase* activity resulting in failure of PCR. In the specific case of ghee (heat clarified milk fat) or heat clarified animal body fat, during heat treatment besides degradation of cell, DNA may also get degraded. Moreover, the degraded DNA gets trapped in the lipid matrix. Thus various methods and kits of DNA isolation were tried and evaluated for isolation of DNA from fat samples, which can subsequently be used for downstream application. Efficiency of DNA isolation was checked by evaluating the quality and quantity of DNA by (i) spectrophotometric method and (ii) PCR using primers. Results of the present study have been discussed under the following heads:

### 4.1.1 Efficiency of Phenol chloroform based method of DNA isolation on the yield of DNA isolated from heat clarified fats

The basic principle of DNA extraction is that all other components of the chromatin are removed leaving behind the DNA. DNA was isolated from heat clarified fat samples by using Phenol chloroform based method as described by De *et a*l. (2011) for isolation of DNA from milk and cheese. Three milliliter(3ml) of melted fat sample was mixed with 3ml of TENS buffer (l0mM Tris, pH 8.0, 1mM EDTA; l00mM NaCl and 0.5% SDS) and incubated at 50°C in a water bath for 30 min with 40 µl of proteinase K for protein digestion. The lower aqueous layer was pipetted out in a 15 ml centrifuge tube. Then, 3ml phenol: chloroform (1:1), 0.8 volumes of isopropanol and 0.1 volumes of 3 M Sodium acetate were added in the tube, after that 700 µl of 70 % ethanol was added and the tube was incubated at -20ºC for 1 hour. Subsequently, the tube was centrifuged at 8,000 rpm for 20 min. Supernatant was removed and precipitate was dissolved in 100 µl 1X TE buffer. To check the quality and quantity of isolated DNA from fat samples, spectrophotometric analysis (Nano Drop spectrophotometer) and 0.8% agarose gel electrophoresis were performed as described under section **3.4.2.1 & 3.4.2.2** of materials & methods.

Amplification behavior of isolated DNA from fat samples was checked as per the methodology described under section **3.5** of materials & methods.

#### 4.1.1.1 Spectrophotometric measurement for quantity and purity of isolated DNA

Genomic DNA is the basic material for application of molecular approaches and the success of such studies largely depend on the quality of DNA isolated. Therefore, to check the quality of DNA, isolated from ghee and heat clarified body fats, a spectrophotometric analysis (**3.4.2.1**) was performed. Quality of genomic DNA means that it should be intact and free from any impurities like RNA, protein, organic solvent or salt. Results of the analysis are depicted in **Table 4.1.** It is evident from the data of spectrophotometric analysis that ratio (260/280) is very low, even the values were in negative and nowhere close to the value of 1.8 which is recommended for high or good quality DNA. Negative 260/280 ratio may also be encountered due to the fact that DNA may be heavily contaminated with other substances that absorb ultraviolet irradiation and therefore impede accurate analysis. Hence, DNA isolated by the above mentioned **Phenol chloroform based method** was neither of good quality nor of suitability for downstream applications.

**Table 4.1: Yield and purity of DNA isolated by Phenol chloroform based method**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | -8.5* | 0.12±0.001 |
| Buffalo Ghee | -0.12* | 0.04±0.010 |
| Lard | 0.4±0.1 | 0.89±0.005 |
| Goat Tallow | 0.32±0.1 | 0.22±0.001 |
| Data represents mean value ± SD (n=3), *= Values in negative so SD not given | | |

Moreover, the quantity of DNA isolated from ghee and heat clarified animal body fat samples was also very low.. Similar findings were reported by Gimenez *et* *al*. (2010), who reported that it is not possible to isolate DNA from oil sample using phenol chloroform method.

### 4.1.1.2 Quality of isolated DNA on 0.8% agarose gel

Some time there is not sufficient DNA (>250ng/ml) to assay spectrophotometric ally, or the DNA may have impurities and therefore impede accurate analysis. A rapid way to estimate the amount of DNA in such samples is to utilize the ultraviolet induced florescence emitted by ethidium bromide molecules intercalated into the DNA. Because the amount of fluorescence is proportional to the total mass of DNA, the quantity of DNA in the sample can be estimated by comparing the fluorescent yield of the sample with that of a series of standards. Thus to check the quality of DNA isolated from ghee and heat clarified animal body fats another test was performed in addition to spectrophotometric analysis. This includes the agarose gel electrophoresis as described in section **3.4.2.2** of materials and methods.

Results of the agarose gel electrophoresis (**Plate 4.1**) clearly demonstrated that no band of nucleic acid was visualized on the agarose gel, which supported the findings of spectrophotometric analysis that DNA was either of very bad quality or was not at all extracted using the **Phenol chloroform based method.**

### 4.1.1.3 PCR of isolated DNA using universal primer

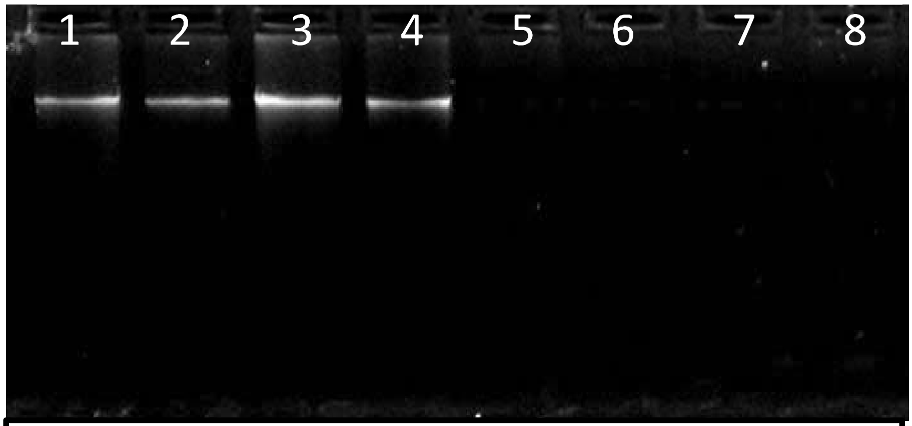
Purity ratios are important indicators of sample quality, nevertheless the best indicator of DNA quality is functionality in the downstream application of interest. Moreover, DNA was not at all detected either by the spectrophotometric method or electrophoresis. Thus, to identify extraction of any DNA molecule, PCR approach was employed which amplifies a single or few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence.

Accordingly, a universal primer pair as listed in section **3.4.3.1** of materials & methods was used to set up a PCR and amplify the DNA isolated from ghee and heat clarified animal body fats. Results of the universal primer-1 (**Plate 4.2)** also suggested that there was no amplification in DNA samples isolated from ghee and heat clarified body fats using phenol chloroform method. The present findings are not in agreement with the results of De *et al*. (2011), this may be due to the fact that they used this method for isolation of DNA from milk and cheese. As milk is a rich source of somatic cells (Lipkin *et al*., 1993), so there it was possible to isolate sufficient quantity of DNA from milk sample. On the contrary, in case of ghee or other heat clarified body fats the chances of somatic cells transfer to 99% triglycerides may be minimal. In addition, there is high possibility of degradation of DNA due to high temperature of clarification i.e. 110-120°C used during ghee manufacture and up to 140°C for clarifying the animal body fats. However, the results of the present investigation are in the accordance with the earlier observations of Sonia *et al*. (2014) who reported that traditional DNA isolation methods (based on phenol chloroform) were not suitable for isolation of DNA from refined vegetable oil samples. Since, phenol- chloroform method was not able to isolate sufficient quantity of DNA for downstream application; hence a second method was investigated for isolation of DNA from ghee or heat clarified body fats.

### 4.1.2 Efficiency of Phenol chloroform and iso-amyl alcohol based method of DNA isolation on the yield of DNA isolated from heat clarified fats

De-proteinisation during DNA extraction is more efficient when two different organic solvents are used instead of one. Thus first method was modified to include both chloroform and iso-amyl alcohol in the step involving only chloroform. chloroform iso-amyl alcohol is a type of detergent. It binds to protein and lipids of cell membrane and dissolves them and intern disrupt the bonds that hold the cell membrane together and cause it to breakdown. It then form complexes with these lipids and proteins, causing them to precipitate out of solution. Lipids and proteins are non-aqueous compounds and DNA/RNA are aqueous compounds. The detergent binds to non-aqueous compound. DNA is insoluble in alcohol so it will floats up and all other cell components will precipitate at the bottom of the test tube. In addition, iso-amyl alcohol prevents the foaming caused by phenol during extraction. The details of the method are as:

Five mililiter (5ml) of liquid fat was mixed in 10 ml of DNA extraction buffer (75 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl (pH 8.0) with 40 µl of proteinase K and 400 µl of SDS (10%). It was incubated overnight at 50º C in a water bath and thereafter, an equal volume of Tris-saturated phenol (pH 8.0) was added and mixed thoroughly. The resultant mixture was centrifuged at 13,000 rpm at 22º C for 10 min and the lower aqueous layer was pipetted out in a 15 ml centrifuge tube. Then, 3 ml of freshly prepared mixture of phenol, chloroform and iso-amyl alcohol (25:24:1) was added, thereafter 1.5 ml of chloroform and iso-amyl alcohol mixture (24:1) was added in that 15 ml centrifuge tube containing that aqueous solution and mixture of phenol, chloroform and iso-amyl alcohol (25:24:1). Thereafter the tube was centrifuged to 5000 rpm / 5 min. Then, the supernatant (500µl) was collected in a sterile Eppendorf tube and the DNA in the supernatant was precipitated with 0.8 volumes (400µl) of isopropanol in the presence of 0.1 volume (50µl) of 3 M sodium acetate. To the precipitate and lower layer, obtained after centrifugation at 12,000 rpm at 4º C for 10 min, 700 µl ethanol (70%) were added followed by centrifugation



1-cow DNA from blood,2-buffalo DNA from blood,3-goat DNA from blood,4-pig DNA from blood,5-cow ghee,6-buffalo ghee,7-lard,8-goat body fat

Plate 4.1 : 0.8% agarose gel electrophoresis for DNA isolated from fat samples

1- 100 bp marker,2 - DNA from blood,3-cow ghee,4 -buffalo ghee,5-lard,6-goat tallow (gt)

Plate 4.2 Amplification of isolated DNA from fat samples using Universal primer-1 at 12,000 rpm at 4º C. After centrifugation all the liquid were pipetted out and discarded and thereafter precipitated DNA were eluted in 100 µl 1X TE Buffer.

#### 4.1.2.1 Spectrophotometric measurement for quantity and purity of isolated DNA

To check the quality of DNA isolated from ghee and heat clarified animal body fats by using Phenol chloroform and iso-amyl alcohol based method, spectrophotometric analysis was performed as described in section **3.4.2.1**. Results of the analysis are depicted in **Table 4. 2.** It is evident from the data of spectrophotometric analysis that ratio of absorbance at (260/280) is negative and nowhere close to the value of 1.8 which is recommended for high or good quality of DNA. A low ratio may be the result of a contaminant absorbing at 280 nm or less which may include residual phenol or other reagent associated with the extraction protocol or it may be due to the very low concentration (<5 ng/ul) of nucleic acid. Therefore, the DNA isolated by the above mentioned **Phenol chloroform iso-amyl based method** was neither of good quality nor of suitability for downstream applications.

**Table 4.2: Yield and purity of DNA isolated by Phenol chloroform and iso-amyl alcohol based method**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | -0.44* | 1.2 ±0.003 |
| Buffalo Ghee | -0.68* | 0.39 ± 0.01 |
| Lard | -0.56* | 0.86± 0.034 |
| Goat Tallow | -0.76* | 0.44 ± 0.01 |

Data represents mean value ± SD (n=3), *= Values in negative so SD not given

#### 4.1.2.2 Evaluation of the quality of isolated DNA on 0.8% agarose gel

It is essential to determine the quality of DNA before use in PCR. Intact, undigested DNA results in a band, whereas a smear indicates degradation. However, no DNA bands, intact or degraded were visible on 0.8% agarose gel electrophoresis as described in section **3.4.2.2**. It can be concluded that either the DNA was not at all been extracted or the concentration of isolated DNA was less than 5ng/µl, that is the minimum DNA concentration to be detected by agarose gel electrophoresis (Sambrook *et al.,* 1989).

#### 4.1.2.3 PCR of isolated DNA using universal primers

To explore the possibility of extraction of very minimum quantity of DNA from the ghee, PCR was set up using universal primer-1 as listed in section **3.5.1** of materials & methods. No amplification was observed after electrophoresing the PCR product on 2% agarose gel electrophoresis.

Spectrophotometric data clearly revealed that DNA isolated from fat sample using this said method was not pure to be used for downstream applications. The findings are in the accordance with the results of Raieta *et al.* (2015), who reported that the amount of DNA isolated from oil or fat samples is also usually low and the quality as well as quantity of isolated DNA was poor as well as degraded. Though Bogani *et al*. (2009), who reported that chloroform and iso-amyl alcohol were the most suitable organic reagent for isolation of DNA from soybean, sunflower, peanut and sesame oil. But in the present study there might be the chances that during heat clarification most of the DNA might have become fragmented and the highly degraded DNA got trapped in between fat matrices. Thus, the said protocol was also not able to purify DNA, with concomitant failure of amplification with universal primer-1, so no amplification was observed on 2% agarose gel during PCR reaction using universal primer-1. In an earlier study it was reported that the degradation of DNA occurred during heat treatment (Ebbehoj and Thomson, 1991). He *et al*. (2013) reported that during simplex PCR no amplification was recorded from DNA isolated from oil sample using chloroform as an extraction solvent. Therefore, in the present study spectrophotometric data, 0.8% gel electrophoresis observation as well as simplex PCR followed by 2% gel electrophoresis data clearly showed that the said protocol was also not suitable for isolation of DNA from ghee or other heat clarified animal body fat samples.

### 4.1.3 Efficiency of Phenol chloroform CTAB based method of DNA isolation on the yield of DNA isolated from heat clarified fats

CTAB method of DNA isolation from refined vegetable oil was reported by Costa *et al.* (2010). The same has been evaluated in the present investigation to isolate DNA from ghee and heat clarified animal body fat samples. The details of the method are as follows:

Thirty milliliter (30 ml) of fat was mixed by magnetic stirring for 1 h with 30 ml of hexane and then with the 5 ml of CTAB buffer (2% CTAB, 0.8 M NaCl, 50 mM Tris-HCl, 1 mM EDTA) 5 ml for another 30 min. The sample was centrifuged at 11,000 rpm for 10 min. The aqueous phase (10 ml) was transferred to a 50-ml tube and thereafter 1 volume (10 ml) of absolute isopropanol, 1/10 Volume (1 ml) of 3 M sodium acetate were added and tube was stored at -20º C overnight. The tube was centrifuged at 14,000 rpm for 20 min, and the liquid phase was discarded. The residue (precipitated during incubation followed by centrifugation) in the tube, was dissolved in 1 ml of 1X TE buffer and 1 ml of Phenol/chloroform/ iso-amyl alcohol (25:24:1), and the mixture was centrifuged at 12,000 rpm for 10 min. After the centrifugation, 800 µl of the upper part of the mixture was transferred to a new tube, followed by the addition of 1 volume (800 µl) of absolute isopropanol and 1/10 volume (80 µl) of 3 M sodium acetate. The tube was incubated at -20º C for 3 hours, followed by centrifugation at 14,000 rpm for 20 min at 4º C. The supernatant was discarded and the residue was dissolved with 700 µl of 70% ethanol, followed by centrifugation at 14,000 rpm for 10 min. Ethanol portion was pipetted out and discarded. Then DNA was dissolved in 50 µl of 1X TE buffer for further analysis. To check the quality and quantity of isolated DNA from fat samples, spectrophotometric analysis (Nano Drop spectrophotometer) and 0.8% agarose gel electrophoresis was performed as described under section **3.4.2.1 & 3.4.2.2** of materials & methods. Amplification behavior of isolated DNA from fat samples was checked as per the methodology described under section **3.5** of materials & methods.

#### 4.1.3.1 Spectrophotometric measurement for quantity and purity of isolated DNA

To check the quantity and quality of DNA isolated from ghee and heat clarified animal body fats, the spectrophotometric analysis was performed. Results of the analysis are depicted in **Table 4.3.** It is evident from the data of spectrophotometric analysis that (260/280) ratio is very low, even the absorbance values are in negative and nowhere close to the value of 1.8 which is recommended for high or good quality of DNA. Abnormal 260/280 ratio usually indicate that a sample is contaminated by residual phenol, guanidine, or other reagents used in the extraction protocol. Inaccurate ratios may also be observed, if the concentrations of extracted DNA is very low (<10 ng/µl). Therefore, the DNA isolated by the above mentioned **Phenol chloroform CTAB method** was neither of good quality nor of suitability for downstream applications. The results of the present investigation are in accordance with the earlier observations of Costa *et al*. (2010), who reported poor quality of DNA, isolated from oil sample using phenol chloroform method.

**Table 4.3: Yield and purity of DNA isolated by Phenol chloroform CTAB method**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | -0.56* | 0.49 ± 0.03 |
| Buffalo | -0.66* | 0.66 ± 0.016 |
| Lard | 0.98± 0.04 | 0.67 ± 0.03 |
| Goat Tallow | 0.46 ± 0.11 | 0.04 ± 0.0001 |
| Data represents mean value ± SD (n=3), * = Values in negative so SD not given | | |

Similarly, Testolin and Lain (2005) reported isolation of 10ng/µl DNA from unrefined vegetable oil by using CTAB extraction process. However, Gimenez *et al*. (2010) were not able to isolate DNA from refined vegetable oil even by increasing the volume of vegetable oil by using same CTAB based isolation process. Therefore, the findings of the present study also revealed that using CTAB method it was not possible to isolate quality DNA from ghee or heat clarified animal body fat.

### 4.1.3.2 Quality of isolated DNA on 0.8% agarose gel electrophoresis

To check the quality of DNA isolated from ghee and heat clarified animal body fats another test as described in section **3.4.2.2** of materials and methods was performed. Results of the agarose gel electrophoresis clearly demonstrated that there was no DNA band on the gel, which supports the findings of spectrophotometric analysis that DNA was of either very bad quality or not at all extracted using the **Phenol chloroform CTAB method.**

#### 4.1.3.3 PCR using universal primer -1

To further confirm the absence/ presence of DNA, a universal primer as listed in section 3.5.1 of materials & methods was used to set up a PCR and amplify the DNA isolated from ghee and heat clarified animal body fats. Results of this parameter also suggested that there was no amplification with DNA isolated from ghee and heat clarified animal body fats samples using **Phenol chloroform CTAB based method.** This may be ascribed to the incomplete removal of CTAB which in turn inhibited PCR reaction (Ziegenhagen and Scholz, 1993), who reported that though the use of SDS or CTAB as lysis buffer able to penetrate fat matrix during isolation of DNA from oil but sometime SDS or CTAB get attached to the secondary metabolite of oil and prevent extraction of DNA with high quality and inhibit PCR reaction. However, the result of the present investigation is contrary to the findings of the Busconi *et al*. (2003), who used CTAB method to ascertain the purity of oil.

Therefore, the spectrophotometric data, 0.8% gel electrophoresis observation as well as simplex PCR followed by 2% gel electrophoresis clearly showed the that the said protocol was not suitable for isolation of DNA from ghee and other heat clarified animal body fat samples.

### 4.1.4 Efficiency of Phosphate buffer solution (PBS) followed by Nucleo Spin-tissue kit based method of DNA isolation on the yield of DNA isolated from heat clarified fats

In this case, a method described by Nooratiny *et al.* (2013) was used to isolate DNA from ghee and heat clarified animal body fat samples. However, instead of Epicentre Master-Pure DNA isolation kit, Nucleo Spin kit for DNA isolation was used in the present investigation as the Epicentre Master-Pure DNA kit was not available with Indian suppliers having rate contract with NDRI. The details of the method are described below:

Thirty mililiter (30 ml) of molten fat was mixed with 30 ml of phosphate buffer solution (PBS pH-7.4) for 5 mins followed by centrifugation at 13,000 rpm for 20 min and then the upper layer was discarded. The lower layer was centrifuged at the same speed for another 20 min and transferred into another 50 ml centrifuge tube followed by the addition of different buffers and solutions provided in the Nucleo spin-tissue kit as per manufacturer's instructions. Finally the contents of the tube were loaded to MN-spin column and centrifuged at 11,000rpm /1min, followed by washing with 500 µl wash buffer BW and then with 600µl wash buffer B5 as mentioned in the standard protocol of MN- tissue kit. Then, the MN- spin column was transferred to a 1.5 ml or 2 ml micro centrifuge tube, and eluted with 50µl buffer EB directly (pre warmed to 70°C) by centrifugation at 13,000rpm/1min. Finally 50µl eluted DNA sample was collected and stored at -20°C for further analysis. To check the quality and quantity of isolated DNA from fat samples, spectrophotometric analysis (Nano Drop spectrophotometer) and 0.8% agarose gel electrophoresis was performed as described under section **3.4.2.1 & 3.4.2.2** of materials & methods. Amplification behavior of isolated DNA from fat samples was checked as per the methodology described under section **3.5** of materials & methods.

### 4.1.4.1 Spectrophotometric measurement for quantity and purity of isolated DNA

To check the quality of DNA isolated from ghee and heat clarified animal body fats, a spectrophotometric analysis was performed as described in section **3.4.2.1**. Results of the analysis are depicted in **Table 4.4.** It is evident from the data of spectrophotometric analysis that ratio (260/280) is again low as was the case in earlier studied methods and nowhere close to the optimum value of 1.8 which is recommended for high or good quality DNA. Therefore, the DNA isolated by the above mentioned protocol of using **PBS followed by Nucleo Spin tissue kit** was neither of good quality nor seems to be of suitability for downstream applications. Moreover, the quantity of DNA isolated from fat samples was also very low. Nooratiny *et al.* (2013) reported 100ng/µl of amplifiable DNA from milk fat using PBS approach. But the findings of the present investigation are not in accordance with their results, which may be attributed to the difference in the type of DNA isolation kit used as well as due to lower number of somatic cells expected to be present in the ghee sample.

**Table 4.4: Yield and purity of DNA isolated by PBS followed by Nucleo Spin tissue kit**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | 0.33 ± 0.04 | 1.3 ± 0.2 |
| Buffalo Ghee | 0.25 ± 0.002 | 1.44 ± 0.16 |
| Lard | 0.89 ± 0.03 | 1.38 ± 0.4 |
| Goat Tallow | 0.97 ± 0.41 | 1.68 ± 0.01 |
| Data represents mean value ± SD (n=3), $^{*}$= Values in negative so SD not given | | |

### 4.1.4.2 Quality of isolated DNA on 0.8% agarose gel electrophoresis

To check the quality of DNA isolated from ghee and heat clarified animal body fats another test was performed in addition to spectrophotometric analysis. This included the agarose gel electrophoresis as described in **section 3.4.2.2** of materials and methods. Results of the agarose gel electrophoresis clearly demonstrated that no DNA could be visualized by Ethidium bromide staining of gel, which supported the findings of spectrophotometric analysis of isolated DNA.

#### 4.1.4.3 PCR using universal primer -1

To further confirm the absence/ presence of DNA, a universal primer as listed in section **3.5** of materials & methods was used to set up a PCR and amplify the DNA isolated from ghee and heat clarified animal body fats. Results of this parameter also suggested that there was no amplification in DNA samples isolated from ghee and heat clarified animal body fats using **PBS followed by Nucleo Spin tissue kit based method.** This may be ascribed to the fact that PBS used in this protocol might not be able to penetrate fat matrix to liberate DNA or to the use of **Nucleo Spin tissue kit** for DNA isolation instead of Epicentre Master-Pure DNA isolation kit used by Nooratiny *et al.* (2013).

Therefore the spectrophotometric data, 0.8% gel electrophoresis observation as well as simplex PCR followed by 2% gel electrophoresis data clearly revealed that the said protocol was not suitable for isolation of DNA from ghee or other heat clarified animal body fat samples.

### 4.1.5 Efficiency of water extraction followed by Nucleo Spin tissue kit mini and DNeasy mericon food kit based methods of DNA isolation on the yield of DNA isolated from heat clarified fats

This approch is based on the procedure described for isolation of DNA from olive oil (Hellebrand *et al.,* 1998). In this case instead of PBS, possibility of using water was evaluated for extracting DNA from ghee and heat clarified animal body fats followed by the purification of DNA by two kits namely **Nucleo Spin tissue kit mini (Macherey- Nagel, Germany**) and **DNeasy mericon food kit (Quiagen, Germeny).** The details of the method are as follows:

Two hundred milliliter (200ml) of melted fat sample was mixed with 100ml of warm (80°C) distilled water. Contents were mixed using magnetic stirrer for 20 min at 60°C. The water extract was separated using separating funnel and concentrated to 2 ml by evaporating water on electric heater. This concentrate was further used to purify DNA using the protocol as described for individual kits i.e. Nucleo Spin tissue kit mini and DNeasy mericon food kit.

### 4.1.5.1 Spectrophotometric measurement for quantity and purity of isolated DNA

To check the quality of DNA isolated from ghee and heat clarified animal body fats, a spectrophotometric analysis was performed. Results of the analysis are depicted in Table 4.5 &4.6. It is evident from the data of spectrophotometric analysis that ratio (260/280) is of absorbance is still very low, even the values are in negative as was the case in previously mentioned other approaches. Therefore, the DNA isolated by the water extract approach was also of not helpful to isolate DNA.

**Table 4.5: Yield and purity of DNA isolated using water extraction approach followed by Nucleo Spin tissue kit**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | -0.43* | 0.50 ± 0.01 |
| Buffalo Ghee | -0.12* | 0.39 ± 0.03 |
| Lard | -0.78* | 0.55 ± 0.12 |
| Goat Tallow | .-0.65* | 0.43 ± 0.13 |
| Data represents mean value ± SD (n=3), *= Values in negative so SD not given | | |

#### 4.1.5.2 Quality of isolated DNA on 0.8% agarose gel electrophoresis

To check the quality of DNA isolated from ghee and heat clarified animal body fats another test was performed in addition to spectrophotometric analysis. This includes the agarose gel electrophoresis as described in section 3.4.2.2 of materials and methods. Results of the agarose gel electrophoresis clearly demonstrated that there was no appearance of any band on the gel plate, which supported the findings of spectrophotometric analysis indicating that DNA was of either very bad quality or not at all extracted using the present protocol.

#### 4.1.5.3 PCR using universal primer -1

To further confirm the absence/ presence of DNA, a universal primer as listed in section 3.5.1 of materials & methods was used to set up a PCR and amplify the DNA isolated from ghee and heat clarified animal body fats. Results of this parameter also suggested that there was no amplification in DNA samples isolated from ghee and heat clarified animal body fats using water extract followed by Nucleo Spin tissue kit and DNeasy mericon food kit based protocols. This may be ascribed to the fact that water used in this protocol might not be able to penetrate fat matrix to liberate DNA or both the kits used for DNA isolation were not suitable for isolating DNA from ghee and heat clarified animal body fat.

The findings of the present study are in the accordance with the result of Rabiei (2013) who reported that damaged DNA, is not properly accessible to DNA polymerase due to improper accessibility of sites of damage. In other words, due to high degradation of DNA, binding sight might not be properly available for the primers and hence no amplification was observed. Therefore, on the basis of the spectrophotometric data, 0.8% gel electrophoresis observation as well as simplex PCR followed by 2% gel electrophoresis data clearly showed that the said protocol was also not suitable for isolation of DNA from ghee or heat clarified animal body fat samples. However, the finding are not in accordance with the reports of Hellebrand *et al.* (1998) who reported ample quantity of DNA from both refined and unrefined vegetable oils using the said method. This difference may be due to the type of fat i.e. ghee and heat clarified animal body fats used in the present investigation instead of vegetable oils as reported by the said workers.

**Table 4.6: Yield and purity of DNA isolated by water extraction followed by DNeasy mericon food kit**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | -0.43* | 0.50 ± 0.01 |
| Buffalo Ghee | -0.12* | 0.39 ± 0.11 |
| Lard | -0.78* | 0.55 ± 0.16 |
| Goat Tallow | .-0.65* | 0.43 ± 0.19 |

Data represents mean value ± SD (n=3), * = Values in negative so SD not given

### 4.1.6 Efficiency of DNeasy mericon food kit based protocol of DNA isolation directly from ghee and heat clarified animal body fats

In this case DNeasy mericon food kit based protocol was directly applied in ghee and heat clarified animal body fat samples. Details of the method are as follows:

Two milliliter (2 ml) of molten fat sample was taken into a 2 ml micro centrifuge tube. Thereafter, 1ml food lysis buffer and 2.5µl Proteinase-K were added. The contents of the tube were then incubated in a thermo mixer for 30 min at 60°C with constant shaking followed by centrifugation at 2,500 rpm for five min. After the centrifugation, 700µl of the clear supernatant was transferred into another micro centrifuge tube containing 500µl of chloroform. The tube was vigorously vortexed for

15 s followed by centrifugation at 13,000 rpm for 15 min. Thereafter, 350µl of the upper layer was transferred into another 2ml centrifuge tube containing 350µl buffer PB. The contents were transferred to QIA-quick spin column placed in a 2 ml collection tube and centrifuged at 13000 rpm for 1 min. After this step, 500µl buffer AW2 was added to the QIA-quick spin column, followed by centrifugation at 13,000 rpm for 1 min. Then, the QIA-quick spin column was transferred in to another 2 ml micro centrifuge tube. Finally for elution of DNA, 50µl buffer EB (pre warmed to 70°C) was added into the QIA-quick column followed by 1 min incubation and then centrifugation at 13,000 rpm for 1 min. Finally, 50µl eluted DNA sample was collected and stored at -20°C for further analysis.

#### 4.1.6.1 Spectrophotometric measurement for quantity and purity of isolated DNA

To check the quality of DNA isolated from ghee and heat clarified animal body fat, a spectrophotometric analysis was performed as described in section 3.4.2.1. Results of the analysis are depicted in (Table 4.7). It is evident from the data of spectrophotometric analysis that ratio absorbance at (260/280) has improved over the earlier approaches. Though it is not as desired, but better than the earlier tried approaches and quantity of DNA isolation has also improved. This may be attributed to the fact that initial treatment with lysis buffer along with proteinase-K treatment followed by centrifugation might have helped in separating fat samples into two phases. As CTAB is an essential component of lysis buffer of DNeasy mericon food kit, thus it was able to penetrate fat matirix and liberate DNA into the lower phase of solution. In earlier studies Muzzalupo and Perri, (2002) also reported that during DNA isolation from oil, proteinase-K treatment was very helpful to prevent DNA damage.

**Table 4.7: Yield and purity of DNA isolated by DNeasy mericon food kit method**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | 1.21 ± 0.23 | 6.2 ± 1.3 |
| Buffalo Ghee | 1.11 ± 0.12 | 4.8 ± 1.01 |
| Lard | 1.48 ± 0.44 | 7.9 ± 1.24 |
| Goat Tallow | 1.53 ± 0.13 | 8.4 ± 1.35 |
| Data represents mean value ± SD (n=3), *= Values in negative so SD not given | | |

### 4.1.6.2 Quality of isolated DNA on 0.8% agarose gel

To check the quality of DNA isolated from ghee and heat clarified body fats the agarose gel electrophoresis was carried out as described in section **3.4.2.2** of materials and methods. Results of the agarose gel electrophoresis clearly demonstrated that there was no appearance of any DNA band on the gel, which may be due to the possibility of damage of DNA during ghee manufacture, which might have led to the formation of smaller fragments of intact DNA. Earlier Costa *et al*. (2010) also reported that the size of DNA isolated from refined vegetable oil varied from 103-106 bp and the small sized fragmented DNA was not visible on agarose gel.

### 4.1.6.3 Amplification behaviour of isolated DNA using Universal primer-1

A PCR was set up with universal primer 1 as listed in section 3.5.1 of materials & methods to amplify the DNA isolated from ghee and heat clarified animal body fats. Results showed that there was no amplification of DNA. This may be due the presence of PCR inhibitors which interfered during the DNA amplification steps during PCR. Moreover, presence of DNA did not guarantee the amplification of DNA. In other words, it can be concluded that for positive PCR, pure inhibitor free DNA is very much needed. Results of the present investigation are in line with the earlier observations of Helena *et al*. (2014), who could not amplify DNA isolated from milk using food kit.

### 4.1.7 Efficiency of Nucleo Spin tissue kit based method of DNA isolation on the yield of DNA isolated from heat clarified fats

In this case, Nucleo Spin tissue kit based method as reported by Costa *et al.* (2010) for refined vegetable oil was used with slight modifications in the protocol for isolation of DNA from ghee and heat clarified animal body fat. The isolated DNA was used for downstream application as such as well as concentrated before application.

(i) Five milliliter (5ml) of fat sample was divided equally in five micro centrifuge tubes of 2 ml capacity each. T1 buffer (1ml) and 25 µL Proteinase K were added to each of the five tubes and incubated at 56°C for 20 min, followed by centrifugation at 11,000 rpm for 10 min. The upper portion of the fat layer from each of the tube was discarded and 200µL of B3 buffer was added to all the tubes and incubated at 70°C for 15min. After completion of incubation, 210 µL of 96–100% chilled ethanol was added to each tube and left for 1 min. The contents of the tubes were loaded to five different column and centrifuged at 11,000 rpm for 1min. Washing of the column was done with 500 µl wash buffer BW followed by 600µl wash buffer B5 as mentioned in the standard protocol of Nucleo Spin- tissue kit. The MN- spin column was then transferred to another 1.5 ml or 2 ml micro centrifuge tube, and eluted with 50µl Buffer EB directly (pre warmed to 70°C) by centrifugation at 13,000 rpm for 1min. Remaining 4 columns were also eluted in similar fashion as mentioned in the previous step, but the eluent was the same EB buffer (≈50µl) which was collected after the elution of first column. This resulted into elution of DNA from 5ml of fat in 50 µl elution buffer and increased yield and concentration of DNA.

(ii) In this approach same isolation protocol as described above in (i) was used. However, in the last step, the eluted DNA was further concentrated from 50 µl to 10 µl using a vacuum concentrator.

#### 4.1.7.1 Spectrophotometric measurement for quantity and purity of isolated DNA

Results of the spectrophotometric analysis are depicted in Table 4.8. It is evident from the data that absorbance (260/280) ratio has improved considerably by following approach ‘i’ as described under section 4.1.7 over the earlier approaches. Data also revealed that quantity of DNA had also improved using the studied approach. Thus, a method was standardized by which good quality DNA can be extracted from ghee & heat clarified animal body fats.

**Table 4.8: Yield and purity of DNA isolated by modified Nucleo Spin tissue kit based method following (i) approach**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | 1.11 ± 0.44 | 7.1 ± 1.22 |
| Buffalo Ghee | 1.23 ± 0.36 | 7.8 ± 0.39 |
| Lard | 1.71 ± 0.11 | 18.2 ± 1.8 |
| Goat Tallow | 1.91 ± 0.39 | 14.2 ± 1.87 |
| Data represents mean value ± SD (n=3), $^{*}$= Values in negative so SD not given | | |

### 4.1.7.2 Quality of isolated DNA on 0.8% agarose gel electrophoresis

To check the quality of DNA isolated from ghee and heat clarified animal body fats another test was performed in addition to spectrophotometric analysis. This includes the agarose gel electrophoresis as described in section 3.4.2.2 of materials and methods. Results of the agarose gel electrophoresis clearly demonstrated that there was no appearance of any DNA band on the gel despite of the increased quantity of extracted DNA which has an improved quality as well. The reasons for this type of observation could be ascribed to the fact that the DNA isolated from ghee and heat clarified animal body fats was denatured and fragmented. Similar type of observations were recorded by Costa *et al.* (2010), who reported that small size fragmented DNA was not visible on agarose gel.

### 4.1.7.3 Amplification using universal primer -1

To further confirm the absence/ presence of DNA, a universal primer as listed in section 3.5.1 of materials & methods was used to set up a PCR and amplify the DNA isolated from ghee and heat clarified animal body fat. It is evident from the results that faint but specific bands corresponding to expected amplicon of 360 bp of targeted region of mitochondria were clearly seen on running the PCR products on Ethidium bromide stained 2% agarose gel (Plate 4.3). The appearance of the band the intensity of band indicated that the amount of DNA was either less or qualitatively degraded. Results demonstrated the feasibility of DNA isolation from fat samples that can be used as downstream molecular application.

Similar types of results have been reported in different food stuffs by other workers. Maudet and Taberlet (2001) reported that silica column based extraction protocol of DNA from food matrix was able to isolate sufficient quantity of DNA for downstream application even without the purification step of DNA.

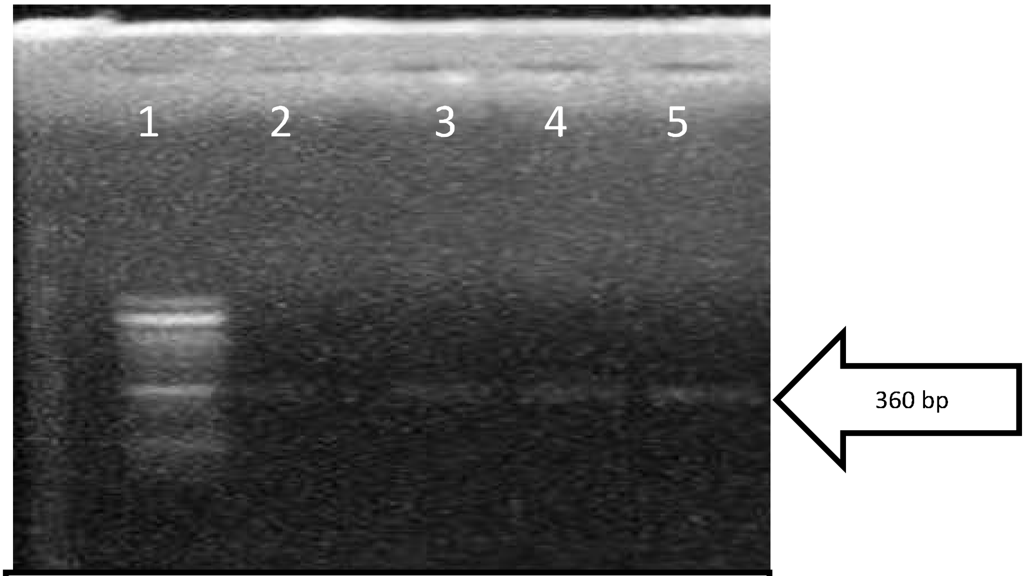
Allmann *et al*. (1992) reported that DNA isolated from bread was around 300 bp long but such highly degraded DNA may still be efficiently amplified by the PCR for amplification. Therefore, on the basis of the spectrophotometric data, as well as simplex PCR followed by 2% gel electrophoresis data clearly stated that the said protocol was suitable for isolation of DNA from ghee or other fat samples. However, it can be concluded that eluted DNA was degraded as well as diluted. Therefore, a second approach was designed to concentrate the isolated DNA before setting up of PCR.

### 4.1.7.4 Efficiency of modified Nucleo Spin tissue kit based method on the yield of DNA isolated from heat clarified fats followed by concentration of the eluted DNA using vacuum concentrator

In this experiment the eluted DNA was further concentrated in vacuum concentrator as stated in approach (ii) under section **4.1.7** before setting up PCR. This attempt was made to overcome the problem of weak amplification of isolated DNA as observed in the previous step.

#### 4.1.7.4.1 Spectrophotometric measurement for quantity and purity of isolated DNA

It is evident from **Table 4.9** that both quality and quantity of isolated DNA improved due to concentration of the eluted DNA. It was contemplated that the approach will lead to sufficient and specific amplification of the DNA by PCR. Due to increase in the isolated DNA upto 19.6 ng/µl, samples were electrophoresed in 0.8% agarose with the expectation that this time DNA can be judged for its quantity and quality. However, on performing the gel electrophoresis on 0.8% agarose, no genomic DNA bands were observed as was the case in previous experiments.



1- 50 bp marker, 2-cow ghee, 3-buffalo ghee, 4-lard, 5-goat tallow

**Plate 4.3: PCR amplification with Universal primer 1 of DNA isolated from fat samples using modified Nucleo-Spin tissue kit method following 'i' approach**

**Table 4.9: Yield and purity of DNA isolated by modified Nucleo Spin tissue kit method following (ii) approach**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | 1.31 ± 0.22 | 9.9 ± 1.2 |
| Buffalo Ghee | 1.76 ± 0.56 | 8.8 ± 1.95 |
| Lard | 1.76 ± 0.47 | 19.2 ± 1.74 |
| Goat Tallow | 1.85 ± 0.33 | 19.6 ± 1.98 |
| Data represents mean value ± SD (n=3), $^{*}$= Values in negative so SD not given | | |

Therefore, the amplification approach based on PCR using the concentrated DNA was also studied in the following experiment to confirm the extraction of DNA as well as the suitability of isolated DNA for molecular applications.

#### 4.1.7.4.2 Amplification using universal primers (1 & 2)

As in case of section 4.1.7.3 of R&D the amplification of the concentrated DNA was done using PCR technique and universal Primer 1. It is evident from **Plate 4.4** that unlike **Plate 4.3**, very sharp and intense bands of the PCR products, corresponding to a specific 360bp was obtained and visualized on electrophoresis in 2% agarose gel. These results clearly demonstrated that by using the above said protocol, it is possible to isolate high quality of amplifiable DNA from Ghee.

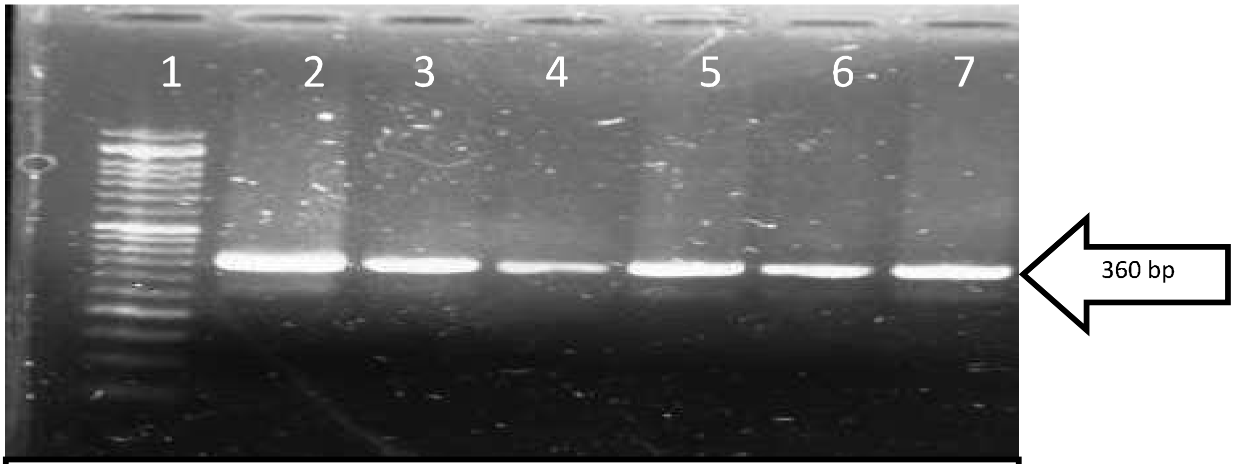
To further confirm the quality of DNA, another primer pair, namely universal primer-2 which targets a different region of mitochondrial DNA was also used to amplify the isolated DNA as per the PCR conditions described in section **3.5.2** of material & methods. Kocher *et al*. (1989) used these two sets of primer (universal primer 1&2) for studying the evolution of *mt-DNA* in various species and concluded that these two sets of primer were able to amplify *mt-DNA* in cattle, goat and pig. Similarly, other workers namely Wong *et al*. (2010) used these primers for identification of species in raw meat products as well as processed meat and meat products. The very reason of using the mt-DNA (mitochondrial DNA) based primers in the present study was that the nDNA (nuclear DNA) tends to degrade faster than mt-DNA. Moreover, the mt-DNA is present in multiple copies compared to nucleus DNA (nDNA) in every cell thus making its detection easier and are likely to remain intact during processing, thereby, minimizing DNA degradation (Woolfe and Primrose, 2004).

It can be seen from (**Plate 4.5**) that DNA was amplifiable and resulted in the expected product of 440bp.

### 4.1.8 Amplification behaviour of DNA isolated from ghee samples procured from market

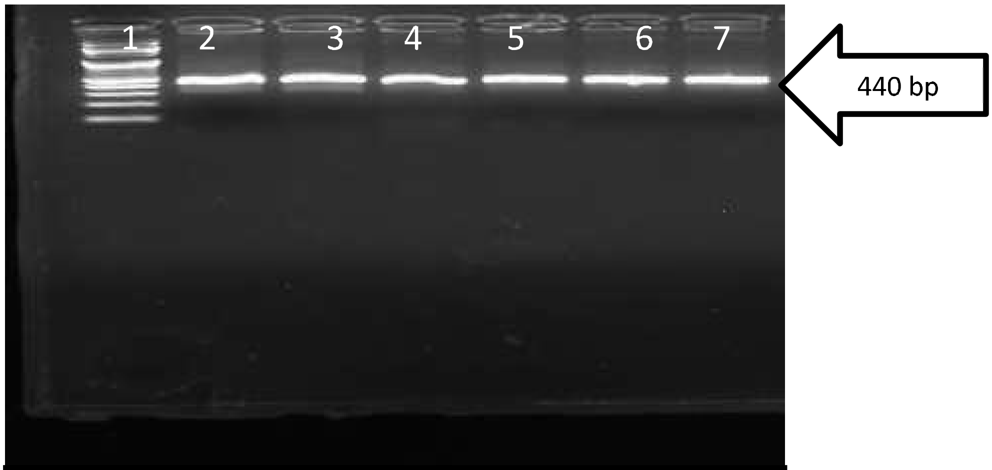
To evaluate the efficiency of the method as discussed in section **4.1.7** of results & Discussion, DNA was isolated from market samples of ghee. The concentrated DNA was subjected to PCR with both the universal primers (1 & 2). The PCR products were run on 2% agarose gel. It can be seen from (**Plate 4.6**) that a product of 360bp, specific for the cyt-b region of mtDNA could be amplified unambiguously from the isolated DNA.

Similarly, PCR with universal primer 2, also resulted in the positive and specific amplification of 440 bp amplicon, corresponding to the desired *12srRNA* gene of mitochondria. The products were specific as concluded from the sharp amplicon bands observed on running the PCR products obtained by amplification of DNA isolated from market samples of ghee (**Plate 4.7**). The detection limit of the *12srRNA*-based PCR system reported in the literature is variable, ranging from less



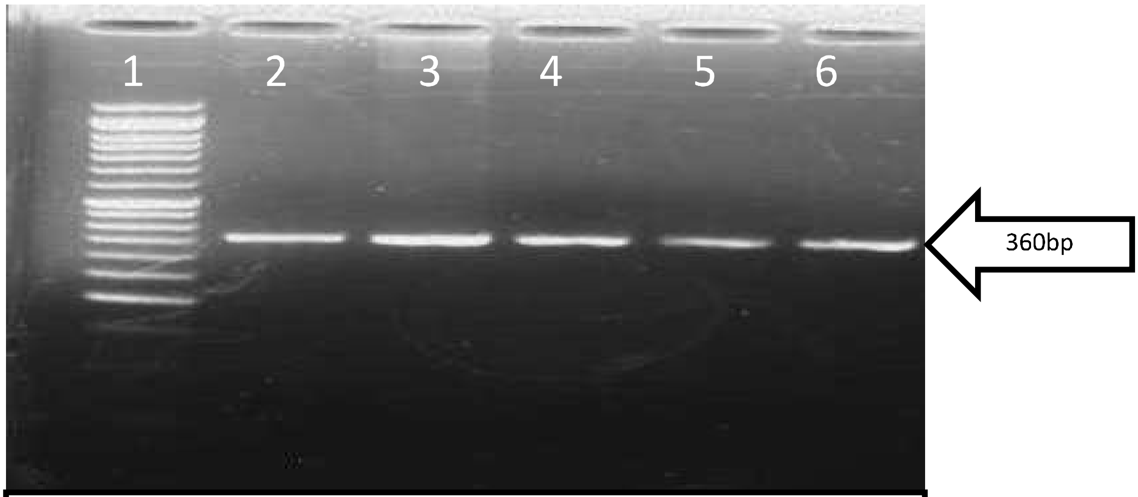
1-50 bp marker, 2-cow ghee, 3-buffalo ghee, 4-goat tallow, 5-lard, 6-DNA from blood of cow, 7-DNA from blood of goat

**Plate 4.4: PCR amplification with Universal primer 1 of DNA isolated using modified Nucleo Spin-tissue kit method following 'ii' approach**



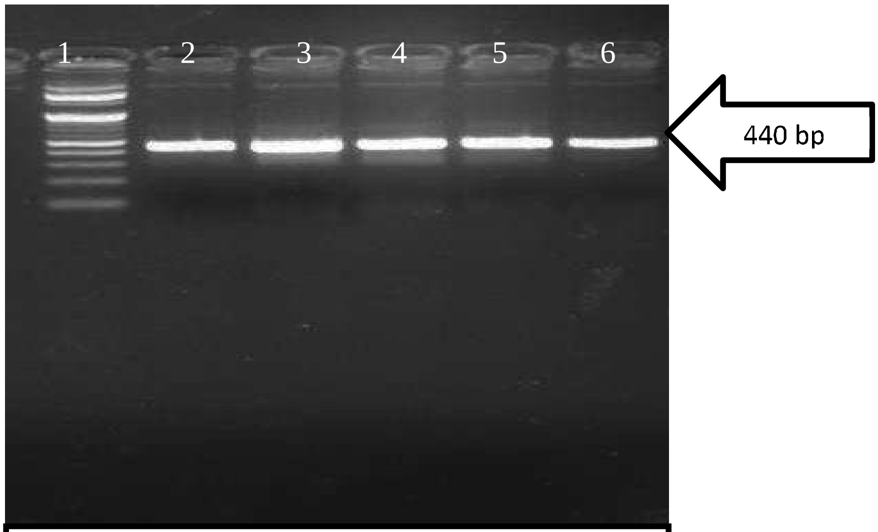
1-100bp marker, 2-cow ghee, 3-buffalo ghee, 4-goat tallow, 5-lard, 6-DNA from blood of cow, 7-DNA from blood of goat

**Plate 4.5: PCR amplification with Universal primer 2 of DNA isolated using modified Nucleo Spin-tissue kit method following 'ii' approach**



1-50 bp marker, 2-6-market samples of ghee

Plate 4.6: Amplification of DNA isolated from market samples of ghee using Universal primer -1



1-100bp marker, 2-6 market samples of ghee

Plate 4.7: Amplification of DNA isolated from market sample of ghee using Universal primer 2

than 5 fg to 10 pg of DNA (Martin *et al*., 2009). However, concentration of DNA by vacuum concentrator is the additional step.

### 4.1.9 Efficiency of modified QIAamp DNA stool kit based method of DNA isolation on the yield of DNA isolated from heat clarified fats

It is evident from the previous discussion that vacuum concentration of DNA was an additional step. Therefore, the method finally standardized was based on the method of Kumar *et al.* (2011a). The modified method has been discussed in section **3.4.1.7** material and methods.

#### 4.1.9.1 Spectrophotometric measurement for quantity and purity of isolated DNA

It is evident from **Table 4.10** that both quality and quantity of DNA isolated from ghee were improved considerably. Data clearly showed that DNA isolated from ghee and heat clarified animal body fats using modified stool kit based protocol has resulted into the DNA of desired 260/280 ratio. This clearly showed that the process could remove the impurities and a good quality of DNA was thus obtained.

**Table 4.10: Yield and purity of DNA isolated by Stool kit based method**

| Fat sample | Purity of DNA (A=260/280) | Quantity of DNA (ng/µl) |
|---|---|---|
| Cow Ghee | 1.66 ± 0.16 | 12.3 ± 1.98 |
| Buffalo Ghee | 1.78 ± 0.11 | 13.8 ± 2.60 |
| Lard | 1.88 ± 0.05 | 21.3 ± 1.35 |
| Goat Tallow | 1.86 ± 0.12 | 23.4 ± 1.47 |
| Data represents mean value ± SD (n=3), $^*$= Values in negative so SD not given | | |

Quantity of DNA was also comparatively good. Isolated DNA can be thus be used for checking the amplification behavior using universal primers as well as for amplification with species specific primers.

#### 4.1.9.2 Amplification of isolated DNA with Universal primers (1 & 2)

It is evident in **Plates 4.8 & 4.9** that DNA of good quality in sufficient quantity has been isolated using the method standardized in the present study. Specific PCR product of 360 bp (**Plate 4.8)** corresponding to the *mt-cytb* gene amplification by universal primer-1 and 440 bp amplicon (**Plate 4.9)** corresponding to the *12s rRNA* gene amplification by universal primer-2 were visible on 2% agarose gel. These results clearly reconfirmed the isolation of amplifiable DNA using this method.

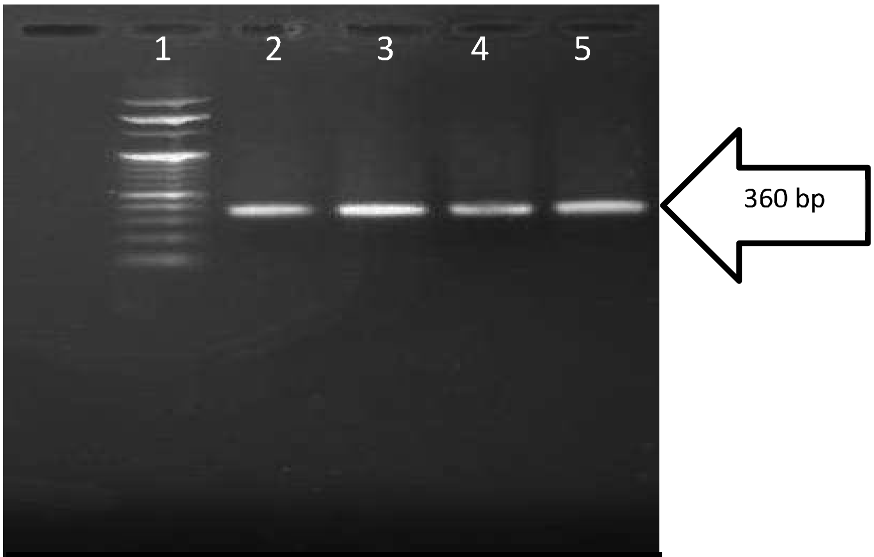
#### 4.1.9.3 Establishing quality of DNA isolated from market sample of ghee

In this case the finally selected method as described in section **3.4.1** of materials & methods was evaluated in market samples of ghee.

It is clearly visible from (**Plate 4.10**) that 360 bp PCR product has been amplified as a result of PCR of isolated DNA using universal primer 1. Similarly, In (**Plate 4.11**) it is clearly visible that a sharp band corresponding to 440 bp amplicon is amplifiable from market samples of ghee. Therefore, it can be concluded that stool kit based protocol was efficient for isolation of DNA from ghee sample prepared in the laboratory as well as for samples procured from local market.

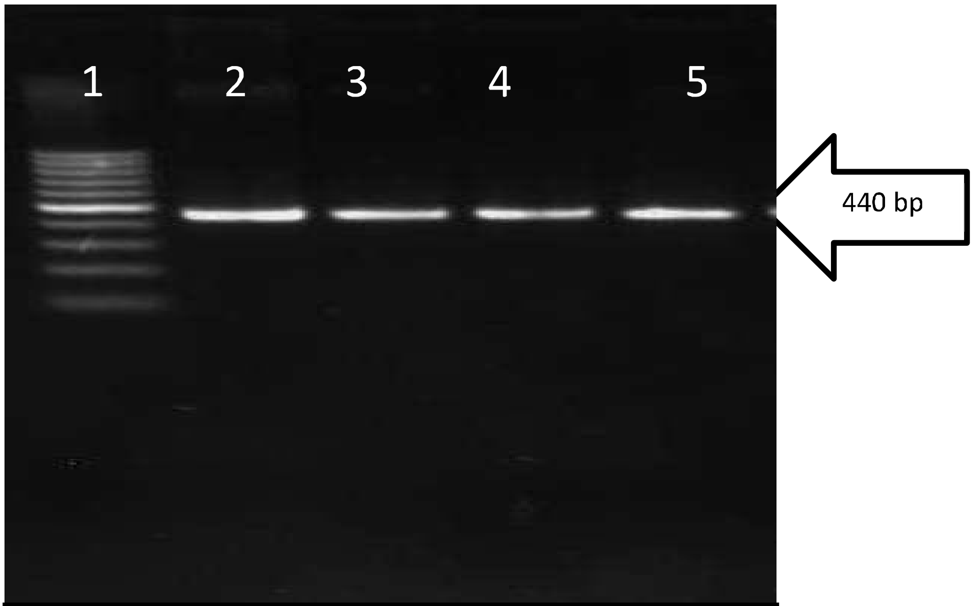
### 4.2 Standardization of a Genomic DNA based method for detection of body fat adulteration in ghee

This section of the results & discussion deals with the standardization of genomic DNA based method for detection of body fat added to ghee as an adulterant. However, the robust and reliable PCR amplification of the highly degraded DNA derived from ghee and heat clarified animal body fats represents a



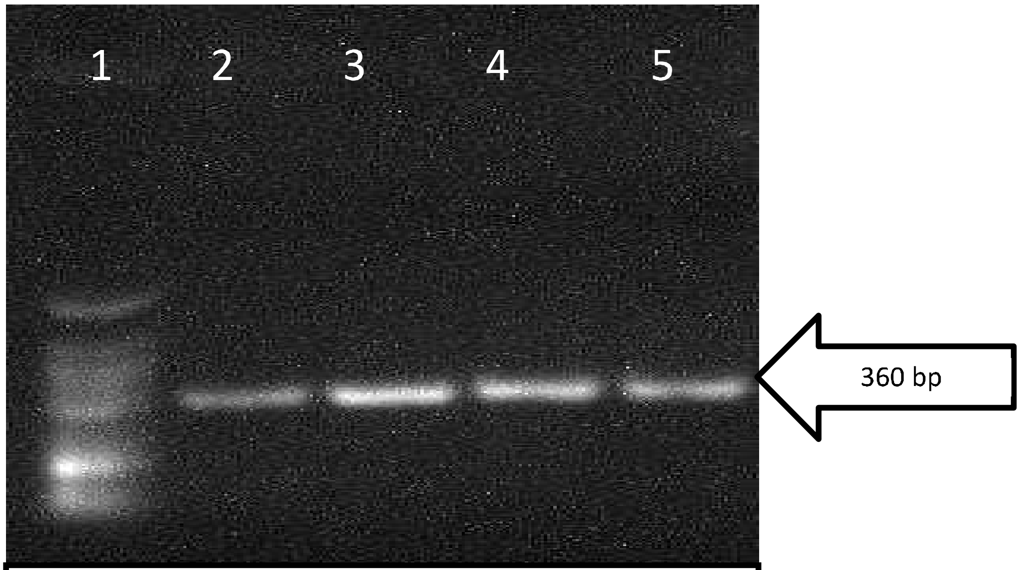
1- 100 bp marker, 2- cow ghee, 3- buffalo ghee,4- lard, 5- goat tallow

**Plate 4.8: Amplification with Universal primer 1 of DNA from fat isolated by Stool kit based method**



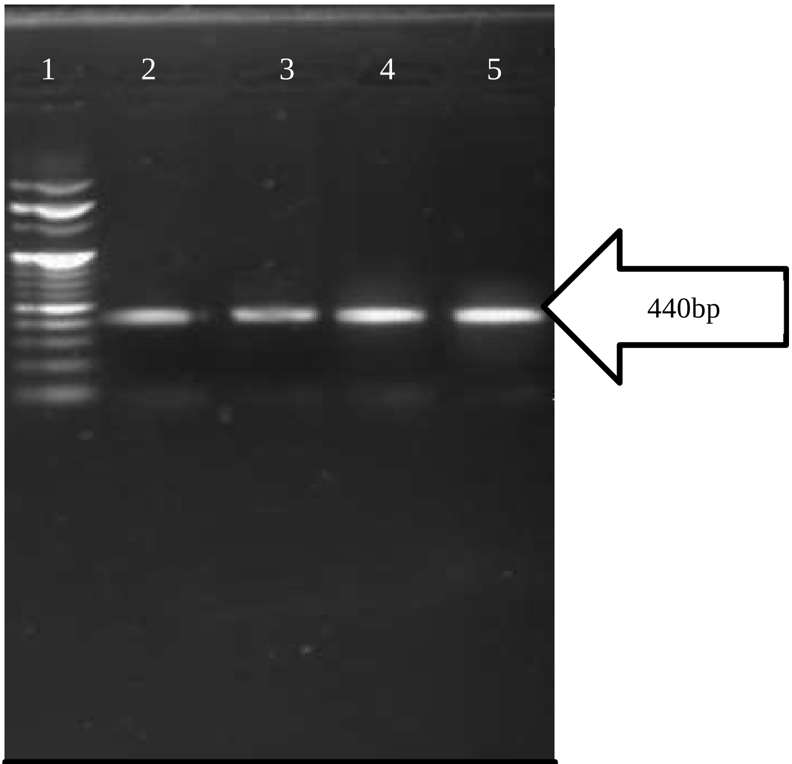
1-100bp marker, 2-cow ghee, 3-buffalo ghee, 4- lard, 5- goat tallow

**Plate 4.9: Amplification with Universal primer 2 of DNA isolated from fat by Stool kit based method**



1:100 bp marker; 2-5:market samples of ghee

**Plate 4.10: PCR amplification of DNA isolated from market sample of ghee with Universal primer -1**



1-100 bp marker; 2-5market samples of ghee

**Plate 4.11: PCR amplification of DNA isolated from market sample of ghee with Universal primer 2**

genuine challenge. However, in **section 4.1**, the feasibility of DNA isolation from ghee and heat clarified animal body fats have been established during this study. Therefore, in this section, ghee samples adulterated with different levels of body fats alone as well as in conjunction with vegetable oils/ fats have been used to check the efficiency of various method of DNA isolation (Section-**4.1.7 and 4.1.9**) followed by species identification of heat clarified animal body fats using species specific primers in simplex PCR. Species specific primers reported in the literature were selected to amplify various regions of targeted gene/genes.

### 4.2.1 Detection of goat tallow in ghee by species specific simplex PCR based approach

#### 4.2.1.1 Selection of literature based goat specific primers

In this experiment, various goat specific primers (**Table 4.11**) as reported in literature were selected to amplify goat DNA isolated from goat blood using conventional method of DNA isolation as described in section **3.6**

**Table 4.11: List of goat specific primers selected from literature**

| Target Gene | Name of the primer | Size of the amplicon (bp) | Reference |
|---|---|---|---|
| *Cyt-b* | T1 | 157bp | Matsunaga *et al*. (1999) |
| *Cyt-b* | T2 | 119bp | Maria-Lopez-Andreo *et al*. (2005) |
| *Cyt-b* | T3 | 404bp | Nagappa *et al*. (2011) |
| *Cyt-b* | T4 | 294bp | Kumar *et al*. (2011b) |

PCR conditions as described in the literature for each primer pair were followed. The results of the species specific simplex PCR clearly showed that primer, T1, T2 and T3 did not amplify goat DNA isolated from goat blood (**Table 4.12**) under the tried conditions

**Table 4.12: Amplification of goat DNA isolated from blood with selected primers**

| Name of the primer | Size of the amplicon (bp) | Results |
|---|---|---|
| T1 | 157bp | × |
| T2 | 119bp | × |
| T3 | 404bp | × |
| T4 | 294bp | √ |
| √= able to amplify DNA , × = Not able to amplify DNA from goat tallow | | |

These results clearly demonstrated that only primer T4 was able to amplify the DNA isolated from goat blood. It was resulted in the desired and expected amplicon of 294bp from goat blood. The same primer was checked for cross amplification with cow and buffalo blood DNA, wherein PCR conditions were kept same as reported by Kumar *et al*. (2011b).

It can be seen clearly in (**Plate 4.12**) that no PCR product corresponding to 294 bp amplicon was observed with genomic DNA isolated from blood of cow. However, with DNA isolated from buffalo blood, two bands were observed with size varying between 200-300bp. The results of the study clearly demonstrated that PCR conditions described by Kumar *et al.* (2011b) may have been optimized to work well in case of meat samples used in their study. When the same conditions were repeated in the present study with DNA isolated from blood, cross reactivity of the primer was observed with buffalo DNA. Therefore, this method was re-standardized and retuned to cater to the needs of the present investigation.

### 4.2.1.2 Standardization of PCR conditions to increase the specificity of primer-T4

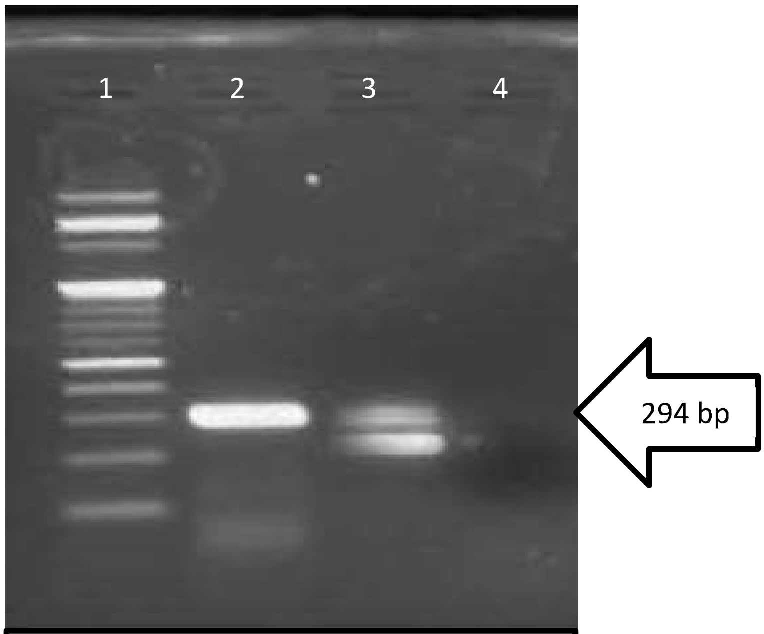
As evident in section (**4.2.1.1)** that there was the issue with the specificity of the primer with DNA isolated from buffalo blood, so to make the method more specific, conditions of PCR were modified. Literature suggests that Magnesium chloride is an essential cofactor for the DNA polymerase in PCR. Its concentration must be optimized for every primer template system. Many components of the reaction bind magnesium ion, including primers, template, PCR products and dNTPs. The main 1:1 binding agent for magnesium ion is the high concentration of dNTPs in the reaction. Because it is necessary for free magnesium ion to serve as an enzyme cofactor in PCR, the total magnesium ion concentration must exceed the total dNTP concentration. In general, magnesium ion should be varied in a concentration series from 1.5 - 4.0 mM with a stepwise increase in concentration @ 0.5 mM. The concentration of $Mg^{2+}$ is dependent on the empirical proportions of template and primer DNA and will need to be standardized for an experiment (Markoulatos *et al.,* 2002). As literature suggests that Magnesium chloride ($MgCl_2$) is a necessary co-factor for all Type II enzymes including restriction endonucleases and polymerases. The divalent cation $Mg^{2+}$ binds to the enzyme inducing a conformational shift that puts the subunits in proper configuration for function. Presumably, a significant reduction in $MgCl_2$ concentration prevents a sufficient number of enzyme molecules from being in the correct conformation for an efficient amplification to occur. Therefore, in the present investigation effect of different concentration of $MgCl_2$ were studied and results have been presented in **Table 4.13.**

**Table 4.13: Effect of different concentration of $MgCl_2$ on the specificity of the goat specific primer T4**

| Concentration of $MgCl_2$ | 1.5mM | 2.5mM | 3.5mM | 4.5mM |
|---|---|---|---|---|
| Cross amplification with buffalo | × | × | √ | * |
| ×= cross amplification with buffalo, √= no cross amplification, * = no amplification | | | | |

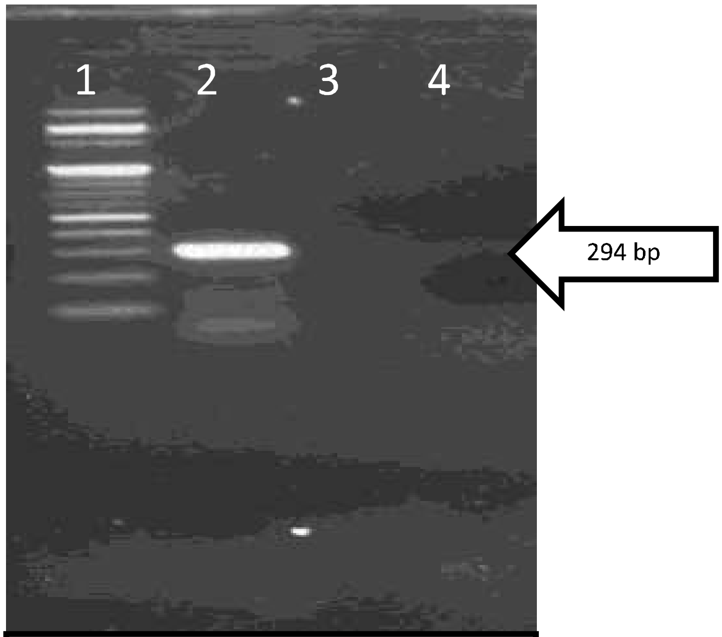
It can be clearly seen that among various concentrations of $MgCl_{2,}$ only 3.5 mM concentration was found to be effective in providing the specificity of the primer in PCR and thereby overcoming the problem of cross reactivity of primer T4 with buffalo blood DNA. It was also observed that beyond 3.5mM concentration of $MgCl_2$ no amplification was observed (**Table 4.13**). These findings were in accordance with the results of Moreira *et al.* (2005), who reported that too much $MgCl_2$ is equally deleterious in most cases. In the case of excess $Mg^{2+}$ the usual effect is a substantial increase in secondary products formation by non-specific priming. Hence 3.5 mM concentration of $MgCl_2$ was selected for setting up the PCR for primer T4.

It is evident from the results that on using 3.5 mM concentration of $MgCl_2$ in the PCR cocktail for Primer T4, there was no band of 294 bp amplicon in the DNA isolated from buffalo blood unlike the one that appeared on using the original PCR conditions (**Plate 4.12**). The 294 bp amplicon was evident only in goat DNA (**Plate 4.13)** and no amplification was observed in the DNA isolated from blood of other species.



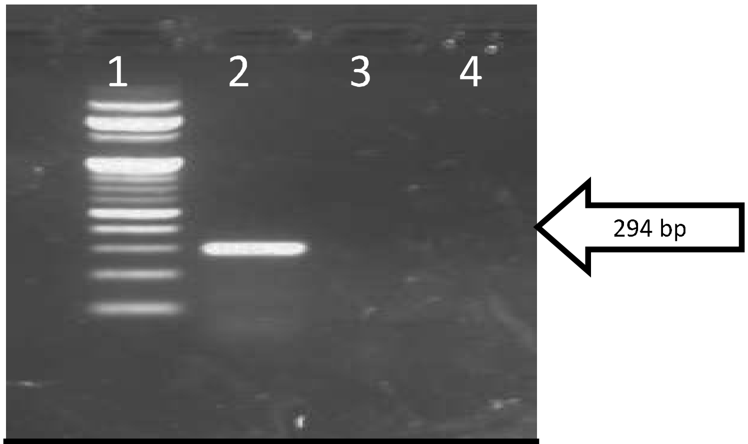
1- 100 bp marker, 2- goat DNA, 3- buffalo DNA,4- cow DNA

**Plate 4.12: Amplification of Goat specific primer (T4) with DNA isolated from blood of different species**



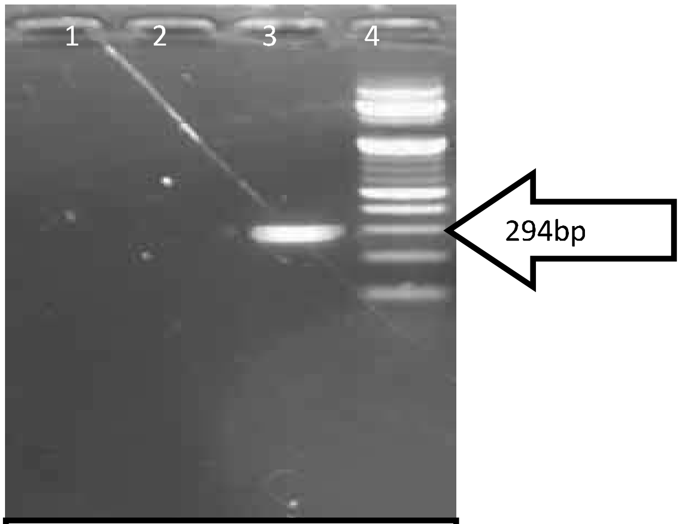
1- 100 bp marker, 2-goat DNA, 3- cow DNA, 4-buffalo DNA

**Plate 4.13: Amplification of Goat specific primer (T4) with DNA isolated from blood of different species**



1- 100 bp marker, 2-goat tallow,
3-cow ghee, 4-buffalo ghee

**Plate 4.14: Goat specific primer amplification with DNA isolated from fat samples by Nucleo Spin Tissue kit based protocol**



1- cow ghee, 2-buffalo ghee,
3-goat tallow,
4-100 bp marker

**Plate 4.15: Goat specific primer amplification with DNA isolated from fat samples by QIAamp DNA stool kit based protocol**

### 4.2.2 Evaluation of specificity of goat specific primer T4 with DNA isolated by modified Nucleo Spin tissue kit based protocol from milk fat (Ghee) and goat tallow

In this case, primer (T4), was evaluated for amplifying DNA that has been isolated from Goat tallow, Cow ghee and buffalo ghee samples using Tissue kit based and Stool kit based protocols, as described in 4.1.7 (ii).

It was clearly visible in (**Plate 4.14**) that there was no amplification observed with DNA isolated form cow or buffalo ghee but 294 bp amplicon was only observed in DNA isolated from goat tallow. Findings of the present investigation clearly established the amplification of mt-DNA and also established that Nucleo Spin-Tissue kit based protocol for DNA isolation from fat samples was able to isolate sufficient quantity of *mt-DNA* from goat tallow for PCR reaction. Similarly, in case of DNA isolated from goat tallow and ghee (cow & buffalo) samples using stool kit based protocol, 294 bp amplicon was observed only in goat tallow samples (**Plate 4.15**).

The results of this experiment clearly demonstrated that both stool kit based as well as Nucleo Spin kit based protocols were efficient in isolating DNA from fat samples in sufficient quantity and good quality. Results of the present investigation reinforced the observation of Cheng *et al.* (2006), that the ubiquitous DNA, from all cell types of an individual species, contains identical genetic information regardless of the origin of the sample**.** However, the results of the present investigation are not in agreement with the findings of Hsieh and Ofori (2015), who reported that adipose tissue was not a good source of DNA. After establishing the possibility of isolation of DNA from goat tallow and species specificity of primer T4, efforts were made to develop a PCR based approach to detect goat tallow in ghee following both Nucleo

Spin-tissue kit and QIAamp stool kit based protocols of DNA isolation and goat specific PCR amplification for ghee samples adulterated with goat tallow.

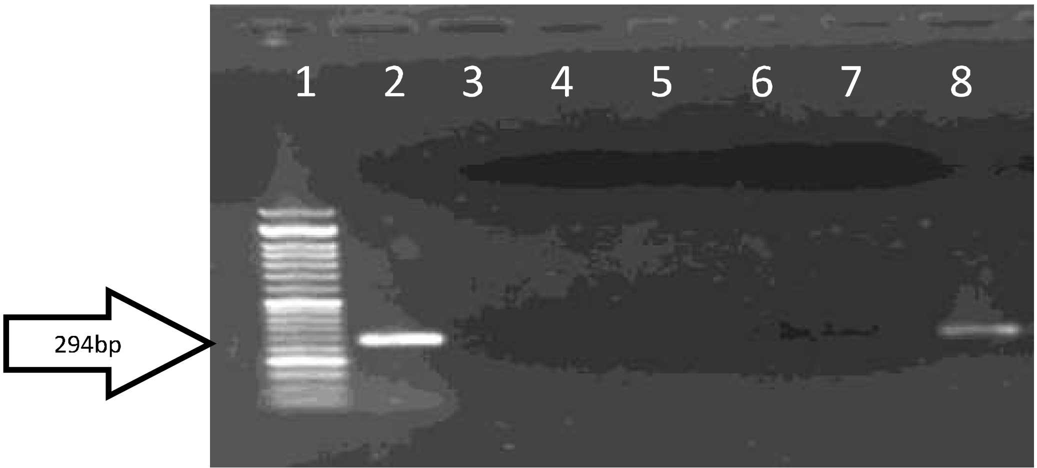
#### 4.2.2.1 Efficiency of standardized PCR method in adulterated samples

DNA isolation protocols namely Nucleo Spin Tissue kit based and QIAamp-DNA Stool kit based were standardized in the present study to isolate DNA from pure goat tallow and ghee samples. These protocols were then further tested in adulterated samples to determine the level of detection of ghee adulteration with tallow.

##### 4.2.2.1.1 Efficiency of Nucleo Spin tissue kit based method of isolation of DNA and PCR to detect goat tallow in ghee

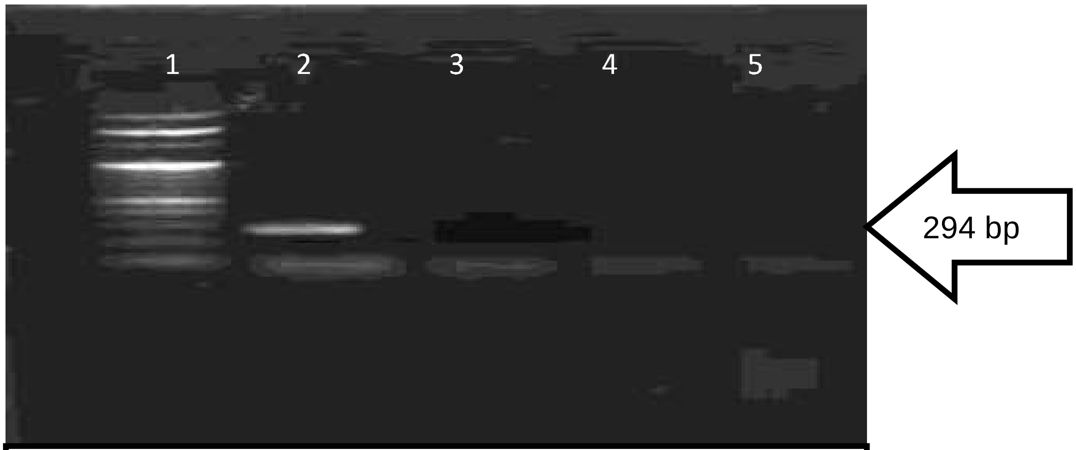
In this experiment the level of detection of adulteration was checked using the standardized method i.e. **Nucleo Spin tissue kit based method** for isolation of DNA and its downstream application in species specific PCR. It is evident from the results shown in (**Plate 4.16 & 4.17**) that in case of ghee samples (Cow & Buffalo) adulterated with goat tallow to the 20% level, sufficient quantity of DNA was isolated which got amplified using goat specific primer resulting into 294 bp amplicon. However, at lower levels of adulteration i.e. 5 & 10% no amplification could be seen for the corresponding 294 bp region of *Cyt-b gene*. Thus it can be concluded that the method used in the study might not be able to isolate sufficient quantity of DNA from goat tallow added to ghee at a level less than 20% and hence subsequent PCR could not result in the amplification of the targeted region.

It can be concluded from the results obtained in the present investigation that **Nucleo Spin** tissue kit based method of DNA isolation could not isolate sufficient quantity of DNA from goat tallow added to ghee at a level less than 20%.



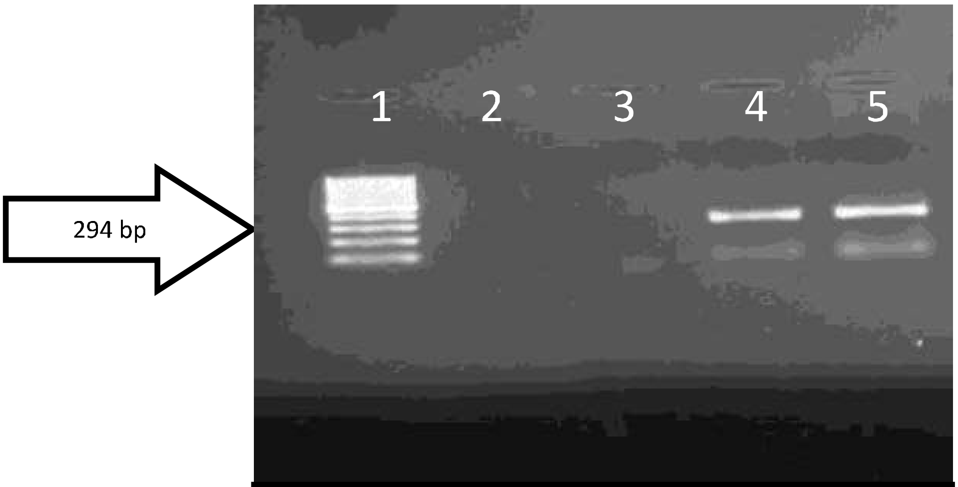
1-50 bp marker, 2-goat blood, 3-cow blood, 4-buffalo blood, 5-pure cow ghee, 6-cow ghee+5% goat tallow, 7-cow ghee+10% goat tallow,8- cow ghee+20% goat tallow

**Plate 4.16: Efficiency of Nucleo Spin-tissue kit based method of DNA isolation and PCR to detect goat tallow in cow ghee**



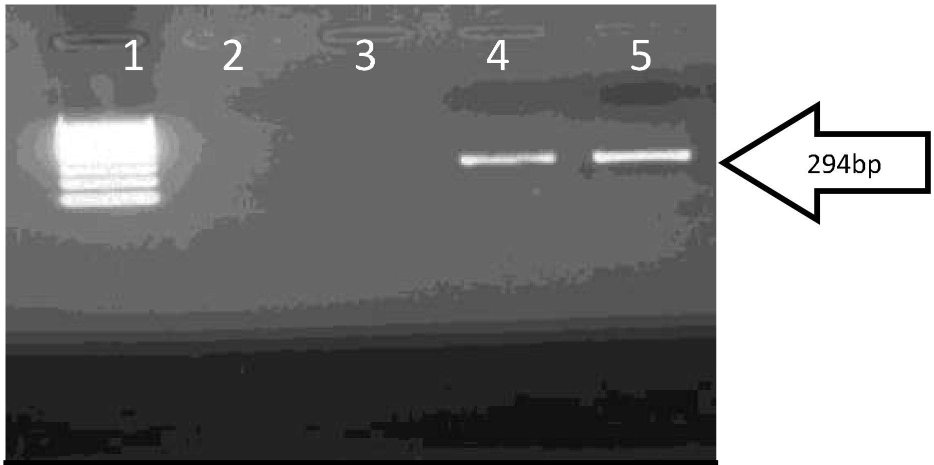
1- 100bp marker, 2- buffalo ghee+20% goat tallow, 3- buffalo ghee+10% goat tallow, 4- buffalo+5% goat tallow, 5- buffalo ghee

**Plate 4.17: Efficiency of Nucleo Spin-tissue kit based method of DNA isolation and PCR to detect goat tallow in buffalo ghee**



1-100bp marker, 2--cow ghee, 3- cow ghee +5% goat tallow, 4- cow ghee +10% goat tallow, 5- cow ghee +20% goat tallow

**Plate 4.18: Efficiency of stool kit based method and PCR to detect goat tallow in cow ghee**



1-100bp marker,2-pure buffalo ghee,3-buffalo ghee+5% goat tallow,4-buffalo ghee +10% goat tallow,5-buffalo ghee +20% goat tallow

**Plate 4.19: Efficiency of stool kit based method and PCR to detect goat tallow in buffalo ghee**

#### 4.2.2.1.2 Efficiency of QIAamp DNA Stool-kit based method of DNA isolation and PCR to detect goat tallow in ghee

In this case efficiency of QIAamp DNA Stool-kit based method in isolation of DNA was evaluated in cow & buffalo ghee samples adulterated with goat tallow.

It is evident from (**Plate 4.18 & 4.19**) that the oligonucleotide primer T4 was able to amplify the goat DNA even in the DNA isolated from ghee sample adulterated with goat tallow following stool kit based method. It can be clearly observed that there was no amplification of DNA isolated from pure buffalo/cow ghee sample.

It is also evident from the results of the study that in case of ghee (cow or buffalo) adulterated with goat tallow @ 5% no band was visible corresponding to a PCR product of 294bp. However, a clear sharp band was visible in case of ghee (cow or buffalo) adulterated with goat tallow @10% level. Hence, this DNA based method was able to isolate sufficient quantity of quality goat specific DNA from ghee having goat tallow to the tune of 10%.

It can be concluded form the results of the present investigation that there was a difference in the level of detection of adulteration of ghee with goat tallow using two different protocols. This might have happened due to amount of fat sample initially used for the isolation of DNA from fats. In case of **QIAamp** Stool kit, it was possible to use large amount (45 ml) of fat sample, whereas in case of **Nucleo Spin** tissue kit only five mililitre of sample was possible to process. This limitation of the use of lesser sample in case of Nucleo Spin- tissue kit based protocol was due to the less quantity of DNA isolation reagents/ buffers available in the kit as the kit has been designed for isolating DNA from tissue samples. Similar kind of reasoning was given by Costa *et al*. (2012), who reported that initial volume of oil or fat sample played a crucial role for possible outcome of DNA from fat samples. Taking into consideration the results of the present investigation, only **QIAamp DNA stool kit** based protocol for isolation of DNA from adulterated ghee samples was further investigated.

### 4.2.3 Efficiency of QIAamp Stool kit based method of DNA isolation in samples of ghee adulterated with admixture of goat tallow and vegetable oil

Detection of animal body fat in ghee is one of the toughest jobs for quality control personnel (Rebechi *et al*., 2016). Detection of body fat in ghee may pose a tough challenge if admixture of vegetable oil and body fat is used as adulterant, which is a rampant practice in India. Therefore, in this experiment the efficiency of the developed method was also checked in ghee samples adulterated with admixture of goat tallow and vegetable oils in such a way so that the effective concentration of goat tallow in the adulterated sample was to the tune of 10%.

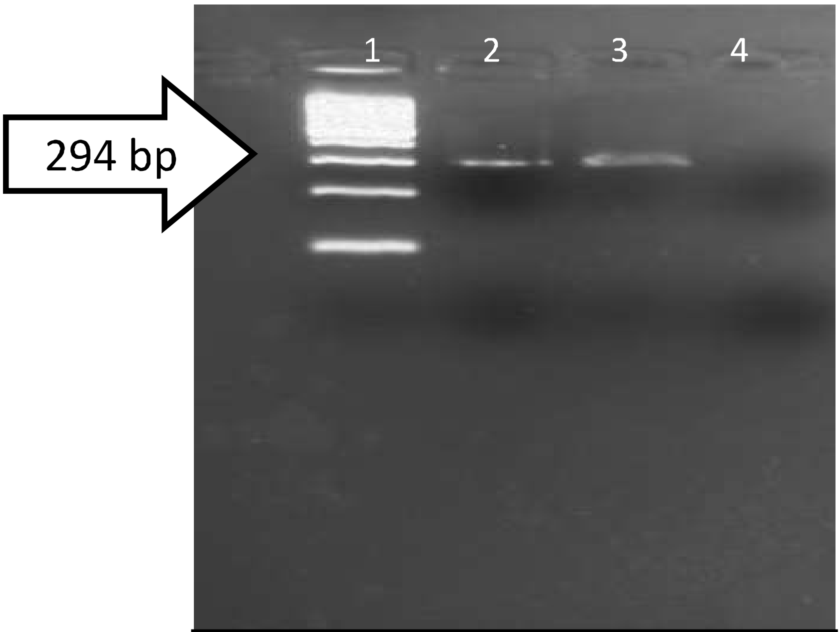
It can be clearly seen in (**Plate 4.20**) that the developed method worked well and adulteration of ghee with admixture of vegetable oils and goat tallow having effective level of goat tallow to the tune of 10% was possible to detect, even in the presence of vegetable oils/fats. Thus it can be concluded that vegetable oils did not interfere with the standardized DNA isolation and amplification protocol.

Findings of the present study are in accordance with the earlier studies, wherein same set of primers was used to detect goat DNA in milk powder and reported the amplification of goat DNA from even highly heated milk powder (Hassan and Ali, 2012).

### 4.2.4. Detection of lard in ghee using species specific PCR approach

#### 4.2.4.1a Selection of literature based species specific primers

In this experiment, various pig specific primers (Table 4.14) as reported in literature were selected to amplify pig DNA isolated from pig blood using



1-100 bp marker, 2- buffalo ghee+10 % goat tallow +10% soy oil, 3- cow ghee+10% goat tallow +10% soy oil,
4- soya oil

**Plate 4.20: Efficiency of stool kit based method and PCR to detect goat tallow in ghee during admixture of animal body fat and vegetable oil**

conventional method of DNA isolation as described in section of 3.6 of materials & methods

**Table 4.14: List of pig specific primers selected from literature**

| Targetd Gene/region | Name of the primer | Size of the amplicon (bp) | Reference |
|---|---|---|---|
| mt-d- loop | L1 | 276bp | Mane *et al.* (2013) |
| *Cyt-b* | L2 | 100bp | Kitpipipt *et al.* (2014) |
| mt-d- loop | L3 | 712bp | Nagappa *et al.* (2014) |
| *Cyt-b* | L4 | 440bp | Matsunaga *et al.*(1999) |
| *Cyt-b* | L5 | 149bp | Dooley *et al.* (2004) |

The results of the species specific simplex PCR (**Table 4.15)** clearly showed that primer L1, L2 and L4 were not able to amplify pig DNA from pig blood following PCR conditions described in the referred research paper. Only primer L4 and L5 were able to amplify pig DNA isolated from pig blood.

**Table 4.15: Amplification of pig DNA isolated from blood with selected primers**

| Targeted Gene/region | Name of the primer | Size of the amplicon (bp) | Results |
|---|---|---|---|
| mt-d loop | L1 | 276bp | × |
| *Cyt-b* | L2 | 100bp | × |
| mt-d loop | L3 | 712bp | √ |
| *Cyt-b* | L4 | 440bp | × |
| *Cyt-b* | L5 | 149bp | √ |
| √=able to amplify DNA , × = Not able to amplify | | | |

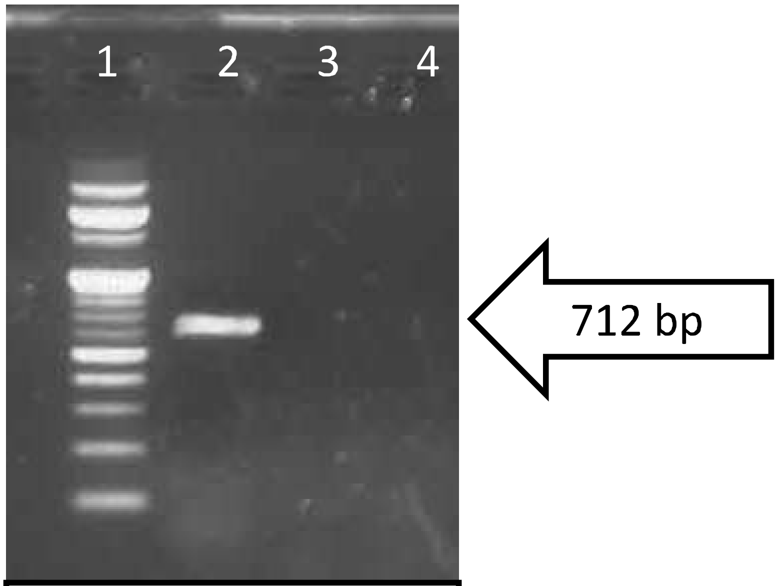
After confirmation for the amplification of two primer sets for pig DNA, these were selected to establish the cross amplification behaviour using DNA isolated from blood of cow and buffalo. Details of the findings have been depicted in **Plates (4.21 & 4.22).**

### 4.2.4.1b Specificity of Pig specific primer (L3 & L5) with DNA isolated from blood of other species

To overrule any interspecies cross reaction, the selected Pig specific primers i.e. **L3 and L5** were used to amplify the DNA isolated from the blood of cow and buffalo.

It is evident from (**Plate 4.21**) that 712bp amplicon corresponding to mitochondrial d loop region was amplified only with the pig DNA on setting up PCR utilizing L3 primer pair. No amplification was observed with the DNA isolated from blood of other species (cow or buffalo). This finding established that the selected primer was very specific to pig DNA under the specified PCR conditions. The results of the present investigation further supported the earlier observation of Nagappa *et al.* (2014), who reported that this selected set of primer was highly pig specific and there was no cross amplification with other species.

It can be seen from (**Plate 4.22)** that 149 bp amplicon corresponding to *Cyt-b* gene was synthesized only with pig DNA on setting up PCR with L5 primer pair. No amplification was observed in the DNA isolated from blood of other species (cow or buffalo). These findings of the present study established that this set of selected primer was also very specific to pig DNA. The results of the present investigation further supported the earlier observation of Dooley *et al*.(2004), who reported that this selected set of primer was highly porcine specific and there was no cross amplification with other species. After conformation of the specificity of the selected



1- 100 bp marker, 2-pig DNA, 3- Cow DNA,
4- buffalo DNA

**Plate 4.21: Specificity of Pig specific primer- (L3) with DNA isolated from blood of different species**

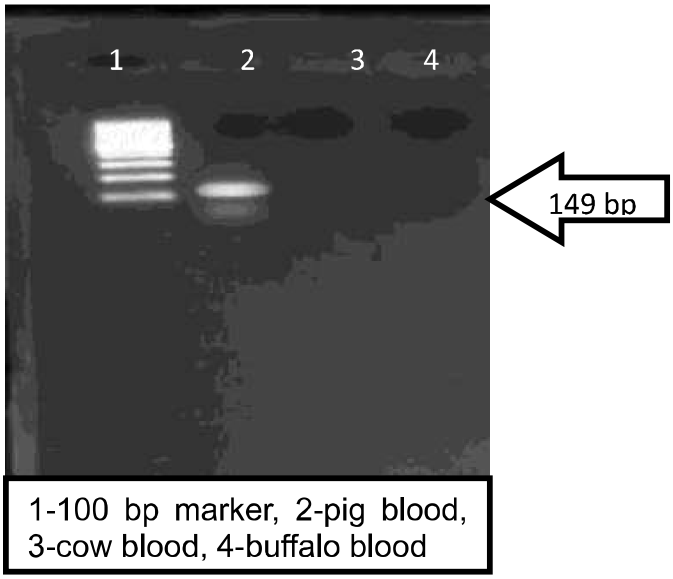


**Plate 4.22: Specificity of Pig specific primer- (L5) with DNA isolated from blood of different species**

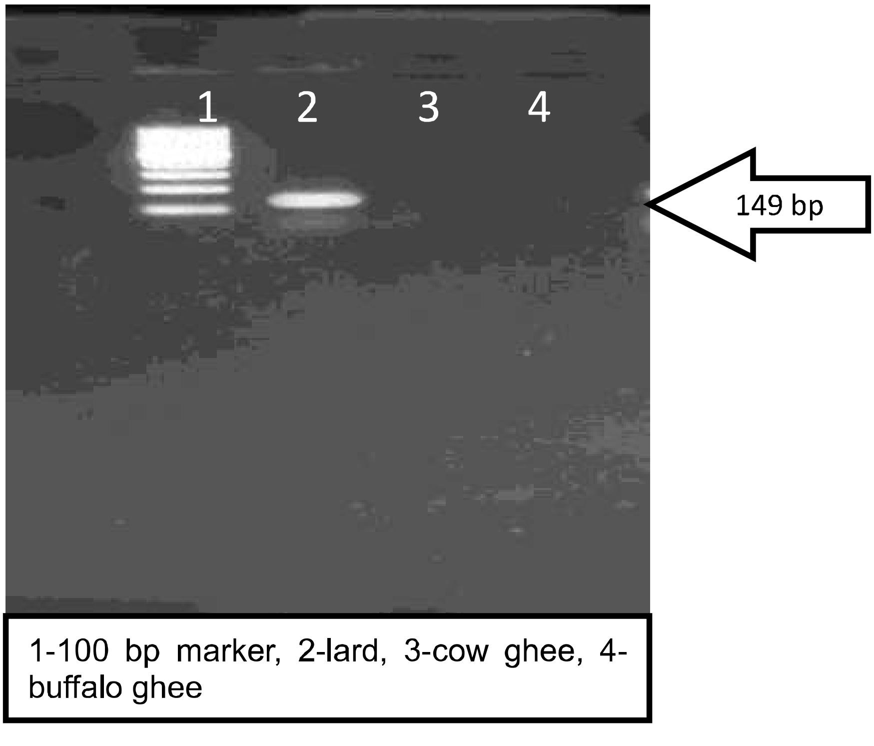


**Plate 4.23: Pig specific amplification with DNA isolated from fat samples isolated by Stool kit based protocol**

primer (L3 and L5) the same set of primers were tried in the amplification of Pig DNA isolated from lard and have been discussed in the subsequent sub sections.

### 4.2.4.2 Efficiency of QIAamp DNA stool kit based protocol to isolate DNA from milk fat and clarified lard followed by species specific PCR

It is evident from preceding discussion that standardized QIAamp **DNA stool kit** based protocol of DNA isolation was more efficient and found to be a suitable methodology to isolate sufficient quantity of DNA from fat samples. Therefore, in order to check the amplification behavior of the selected set of primers in DNA isolated from lard, the said method of DNA isolation was adopted.

#### 4.2.4.2.1a Amplification behavior of Primer L3 with DNA isolated from lard

Results showed that there was no amplification with DNA isolated from lard on setting up a species specific PCR using primer L3. On the contrary this primer was able to amplify PCR product in case of pig DNA isolated from blood. This may be attributed to the fact that nucleic acids isolated from ghee and heat clarified body fats are expected to be severely degraded and may contain mainly small fragments. These fragments represent a poor substrate for molecular biological methods, e.g. PCR (Blow, 2007; Bereczki *et al*., 2007). This may be true with the DNA isolated from lard. The smaller sized DNA fragments may result in the separation of complementary binding sites of forward and reverse primers on two different segments. This will result into failure of amplification during PCR. Similar type of observations were made by earlier workers (Dooley *et a*l., 2004, De *et al.,* 2011, Mane *et a*l., 2013). Thus they used primers targeting smaller region of DNA to identify origin of food products from animal sources. They reported that during high heat treatment, DNA became fragmented in processed food, so specific gene also gets fragmented that cannot bind with specific site of the primer pairs. Similar suggestions were given by Costa *et al.* (2010), who recommended use of short fragmented primer to identify oil species by PCR reaction. Since, this primer was not able to form any PCR product of the specified size in DNA isolated from Lard, therefore the said primer was dropped from the study.

#### 4.2.4.2.1b Amplification behavior of Primer L5 in DNA isolated from lard

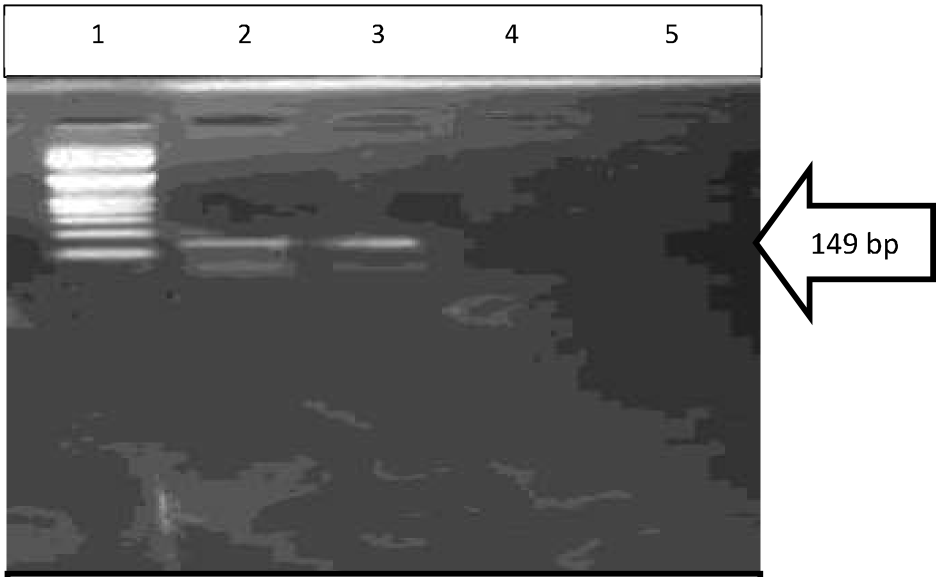
It is clearly visible in (**Plate 4.23**) that amplification was not observed in DNA isolated from cow or buffalo ghee whereas, 149 bp amplicon was observed for the DNA isolated from lard only. These findings also established that the *mt-DNA* isolated from lard had been amplified, whereas DNA from other species could not be amplified. Results also suggested that the selected primer had very high specificity for porcine DNA isolated either from blood or from lard and did not show any cross reactivity with DNA isolated from cow and buffalo ghee.

On the basis of all the above observations, the oligonucleotide primer L5 was finally selected to establish presence of any porcine DNA in the DNA isolated from ghee. Presence of pig DNA was confirmed unambiguously in ghee sample adulterated with lard. It can be clearly seen that there was no amplification of DNA isolated from pure buffalo/cow ghee samples (**Plates 4.24 &4. 25**).

### 4.2.4.3 Efficiency of QIAamp DNA stool kit based method and PCR to detect lard in ghee

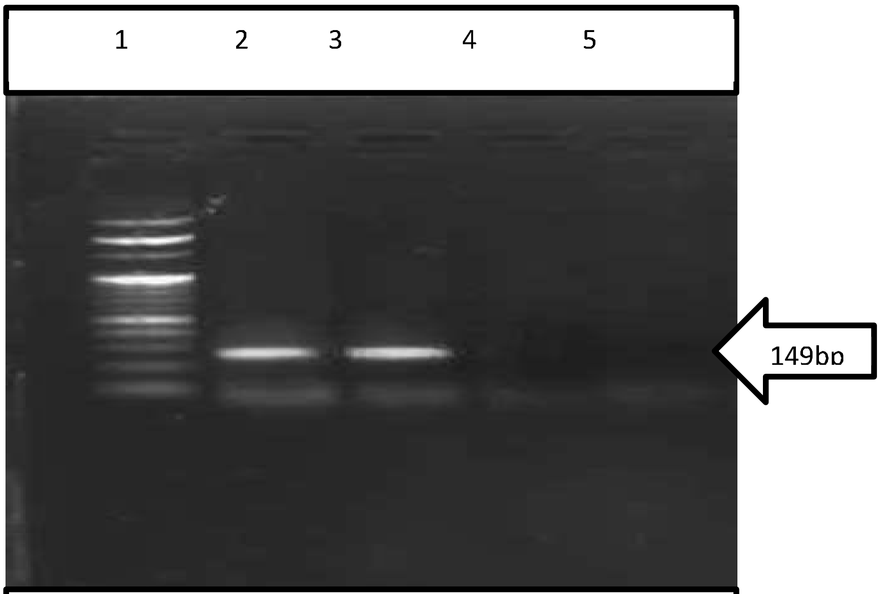
In this case efficiency of QIAamp Stool-kit based method of isolation of DNA was evaluated in cow & buffalo ghee samples adulterated with lard at different levels (5- 20%).

Results obtained clearly showed that the oligonucleotide primer L5 was able to establish presence of porcine species in the DNA isolated from ghee sample



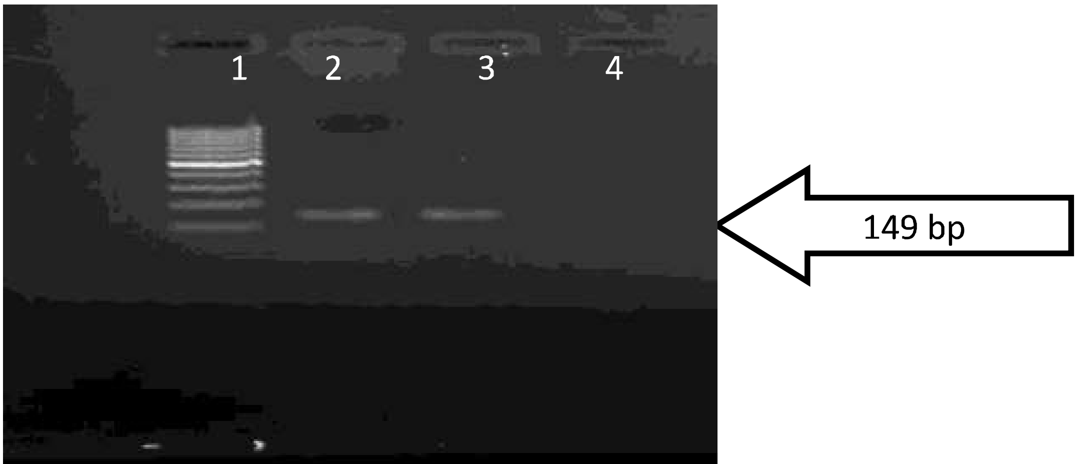
1- 100bp marker, 2-cow ghee+20% lard, 3-cow ghee+10% lard, 4-cow ghee+ 5% lard, 5-pure cow ghee

**Plate 4.24: Efficiency of stool kit based method and PCR to detect lard in cow ghee**



1-50 bp marker,2-buffalo ghee+20%lard,3-buffalo ghee+10%lard,4-buffalo ghee+5% lard,5-buffalo ghee

**Plate 4.25: Efficiency of stool kit based method and PCR to detect lard in buffalo ghee**



1-100 bp marker,2-cow ghee+10%soy oil+10%lard,3-marker,2-buf ghee+10%soy oil+10%lard,4-soy oil

**Plate 4.26: Efficiency of stool kit based method and PCR to detect lard in ghee during admixture of vegetable oil (soy) and lard**

adulterated with lard to the tune of 10% - 20% (**Plates 4. 24 & 4.25**). The level of detection was found to be minimum 10% i.e. ghee (cow/ buffalo) adulterated with lard to the tune of 10% minimum was possible to detect. These findings also proved that using PCR based methodology, it is possible to confirm the presence of lard in ghee.

#### 4.2.4.4 Efficiency of QIAamp DNA stool kit based method and PCR in samples of ghee adulterated with admixture of lard and vegetable oil

As stated earlier in **section (4.2.3)** that it is more difficult to ascertain the quality of milk fat especially ghee, when unscrupulous people add admixture of vegetable oil and body fats as adulterant. Addition of concoctions creates havoc in testing methodologies. Therefore, in the present study efficiency of the developed method was also checked in ghee samples adulterated with admixture of lard and vegetable oil (Soy oil), wherein effective level of adulterant lard was 10%. Results of this study have been depicted as follows:

In (**Plate 4.26**) it can be seen that a band corresponding to 149 bp amplicon has appeared in ghee samples adulterated with a concoction of vegetable oil and lard, wherein effective concentration of lard was @10% level. Therefore, the standardised protocol was found to be suitable to detect lard in ghee and was applicable in situations wherein vegetable oil has been used in conjunction with lard.

The present investigation has successfully demonstrated that it is feasible to isolate sufficient quantity of DNA from ghee and heat clarified animal body fat. The work also led us to the development of protocol for the confirmation of lard and goat tallow in ghee.

### 4.3 Standardization of GLC condition for triglycerides (TGs) analysis

Triglyceride determination has been used to detect foreign fats in milk fat (Precht *et al.*, 1991; Contarini *et al.,* 1993 and Kala, 2013). Also profiling of triglyceride using GLC has been reported to be more effective than fatty acid analysis (IDF & ISO 2010) but the method suggested by IDF & ISO (2010) has not been studied systematically in Indian conditions. Considering the diversity in the types of milk fat a detailed profiling of triglycerides (TGs) is required. Therefore, an effort has been made to differentiate the ghee and adulterated ghee on the basis of trilglyceride profile using GLC. During the study a method reported by Kala (2013), which was based on IDF & ISO (2010) method was selected and evaluated.

During initial phase of the study we used the condition mentioned by Kala (2013), the initial oven temperature of 200 °C was increased to 325 °C at the rate of 5 °C/min and hold at the final temperature for 10 min, the injector and detector temperatures were 330 and 360°C. The reference standard was run under these conditions of GLC analysis.

It is evident from **Plate 4.27** that using the condition mentioned by Kala (2013) only 3 -TGs were clearly resolved instead of five present in the reference standard. Moreover, despite of pre conditioning and repeated cleaning of the column a stable base line could not be obtained as evident from **Plate 4.27**. This might be due to the difference in columns make as Kala (2013) used a HP-5, capillary column of 2.5 m length cut from column of dimension (15 m × 0.25 mm ×0.25 µm) but in the present investigation BP5, capillary column of 2.5 m length cut from a column dimension of (15 m X 0.25 mm X 0.25 µm) was used. Literature also suggested that reported that GLC column plays an effective role for lipid analysis and column specifications were important criteria for lipid analysis (Precht, 1990 and Destaillats *et al.*, 2006). Since,

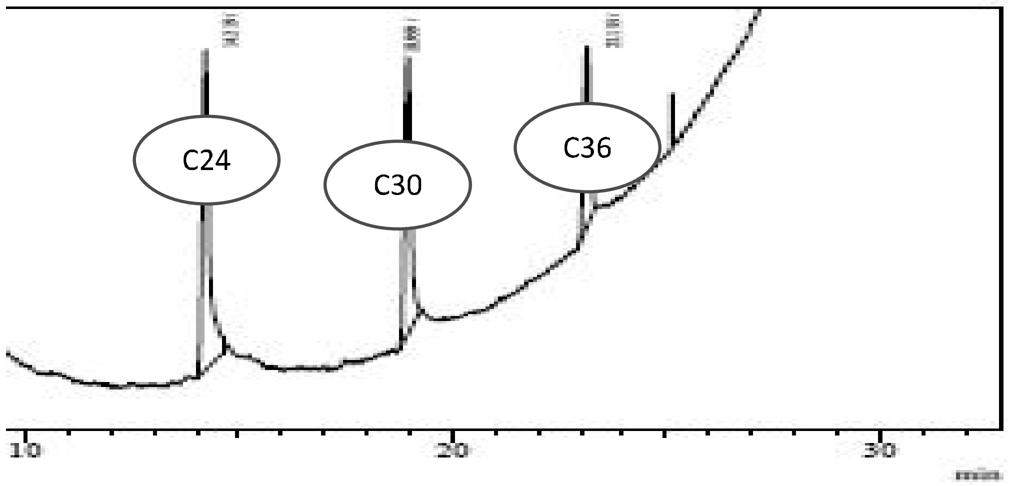


**Plate 4.27: GLC Chromatogram of 5 Triglycerides (C24,C30,C36,C42,C48) reference standard mix run using condition spcified by Kala (2013)**

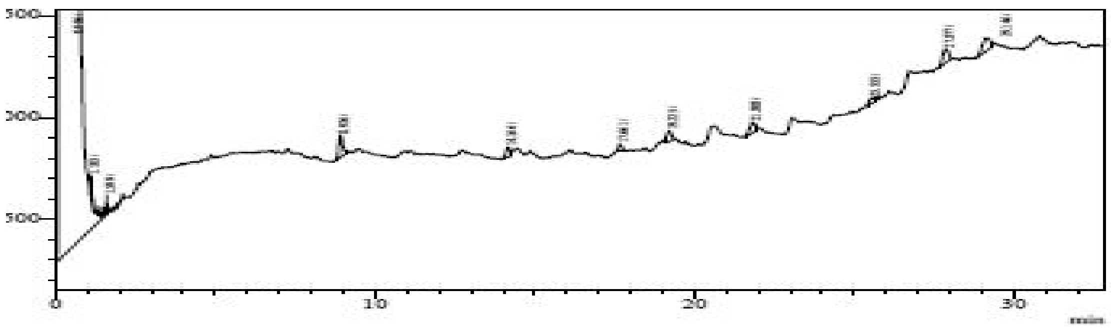


**Plate 4.28: GLC Chromatogram of Triglycerides (C24,C30,C36,C42,C48) reference standard mix with using split ratio (1:20)**

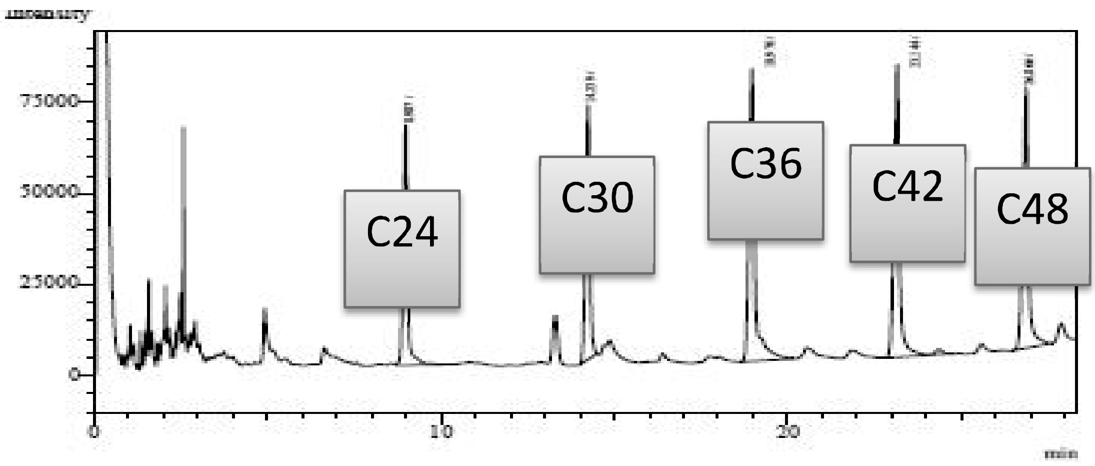


**Plate 4.29: GLC Chromatogram of Triglycerides (C24,C30,C36,C42,C48) reference standard mix with using split ratio (1:50)**

the resolution of triglycerides was poor using the conditions specified by Kala (2013), So GLC conditions like temperature programming, make up volume and flow rate of gas etc. were standardized so that all the five component triglycerides in the reference standard are eluted at different retention times.

It is evident from (**Plate 4.28**) that on using a programme as specified in section 3.8.1.9 of materials & methods all five TGs in the reference standard were clearly eluted at different retention time and base line was also stable (**Plate 4.29**). To check the repeatability of the results using standardized GLC conditions, reference TG standard was injected five times and every time retention times were recorded (**Table 4.16**). Finally a programme of total 32.87 min wherein sample was injected at an initial temperature of 80°C maintained for 0.50 min and then raised temperature with a ramp rate of 50°C/min to 190°C by holding at this temperature for 1 min, then again raised up to 335°C with ramp rate 6°C/min and hold at that temperature for 5min.and split ratio (1: 50) was standardized and used in the present investigation.

**Table 4.16: Retention time of five Triglycerides of reference standard mix**

| TGs | Retention time |
|---|---|
| C24 | 9.01± .05 |
| C30 | 14.34±0.04 |
| C36 | 19.23±0.10 |
| C42 | 23.42±0.04 |
| C48 | 27.01±0.02 |
| Data represent average value of retention time ± SD value (n=5) | |

### 4.3.1 Triglycerides composition of pure cow and buffalo ghee

There were 16 major peaks (**Plate 4.30 & 4.31**) of even carbon number TGs, corresponding to TG of 24 to 54 carbon number, in both cow or buffalo milk ghee. However some unidentified peaks were also observed which might be of odd carbon number TGs but areas of those TGs were very low.

In cow ghee six TGs having carbon numbers (C36, C38, C40, C42, C50 & C52) were predominant (**Table 4.17**). Concentration of these six TGs was almost sixty percentage of total TG present in cow ghee. Presence of C24 carbon number TG was almost negligible **Table 4.17** in cow ghee. Smaller carbon number TG like (C26, C28, C30, C32) found to be in very small amount (**Table 4.17**), whereas TG having carbon number C34 - C54 were the major TGs. It was also noted from **Table 4.17** that in both cow and buffalo ghee there were two clear maxima, located at C38 and C50 **–** C52. Almost similar observations were reported by Fontecha *et al*. (1998). It was also observed from **Table 4.17** that quantity of TGs C50 & C52 were almost similar. Results in the present study are in accordance with earlier observations of Kim *et al*. (2015) and Kala *et al*. (2013) who reported that the major TGs of cow milk fat were C30 to C54. Destaillats *et a*l. (2006) who also identified 16 TGs peaks (C24-C54) of pure cow milk fat on GLC using packed column.

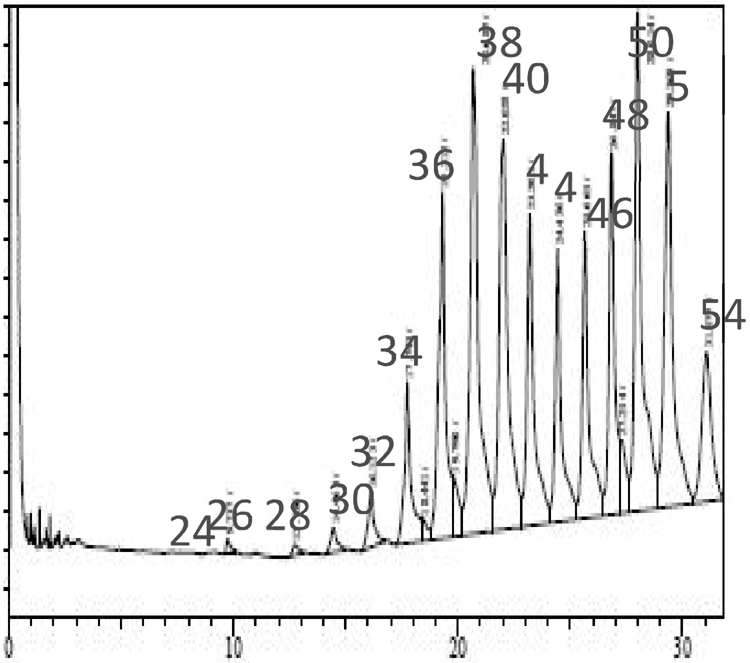


Plate 4.30: GLC Chromatogram of pure cow ghee Triglycerides profile

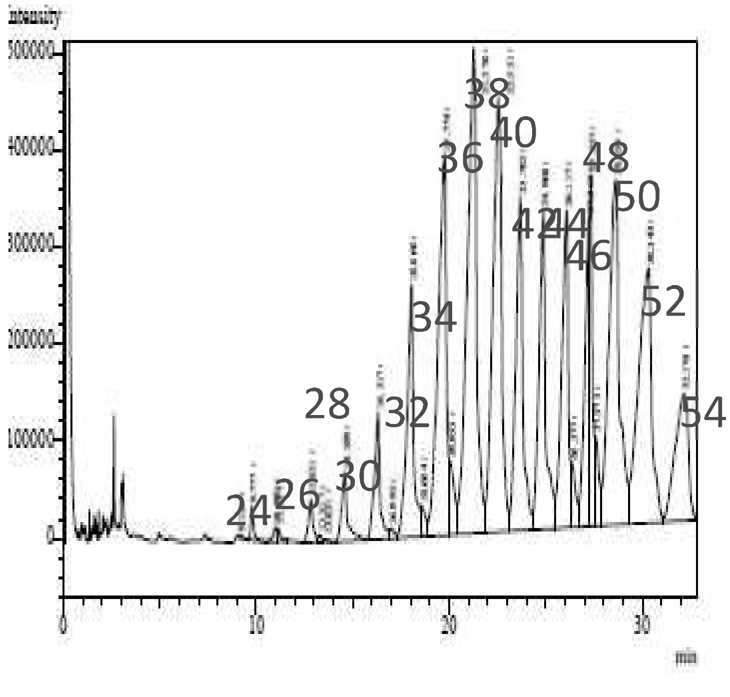


Plate 4.31: GLC Chromatogram of pure buffalo ghee Triglycerides profile

**Table 4.17: Triglycerides (percent w/w) profile of Cow ghee & Buffalo ghee**

| TGs | Cow Ghee | Buffalo Ghee |
|---|---|---|
| C24 | 0.075±0.001 | 0.086±0.003 |
| C26 | 0.33±11 | 0.39±0.15 |
| C28 | 0.35±0.1 | 0.64±0.1 |
| C30 | 0.57±0.19 | 1.02±0.2 |
| C32 | 1.41±0.18 | 2.51±0.32 |
| C34 | 3.68±1.3 | 4.85±0.12 |
| C36 | 9.25±0.8 | 10.64±0.9 |
| C38 | 12.85±0.89 | 13.62±1.14 |
| C40 | 11.92±0.36 | 11.44±1.36 |
| C42 | 6.98±0.2 | 7.017±0.38 |
| C44 | 6.15±0.3 | 5.81±0.86 |
| C46 | 6.14±0.5 | 6.08±0.54 |
| C48 | 7.85±0.8 | 6.97±0.48 |
| C50 | 11.81±1.12 | 10.02±1.25 |
| C52 | 11.61±1.1 | 9.88±1.1 |
| C54 | 6.13±0.5 | 4.35±.85 |
| Data represents average value± SD value (n=6) | | |

Similarly, in buffalo ghee there were six predominant TGs having carbon numbers (C36, C38, C40, C42, C50 & C52) (**Table 4.17).** These six TGs were more than sixty percentage of total TGs present in buffalo ghee. Presence of C24 carbon number TG was almost negligible (**Table 4.17**) in buffalo ghee. Smaller carbon number TG like (C26, C28, C30, C32) found to be very nominal (**Table 4.17**) as in case of cow ghee whereas TGs with carbon chain length of C34-C54 were the major TGs identified in buffalo ghee also.

### 4.3.1.1 Comparison between triglycerides profile of cow and buffalo ghee

From **Table (4.17)** it is clearly visible that for both cow as well as for buffalo ghee C24-C54 TGs were major TGs identified. It is also evident from **Table 4.17** that the quantity of TG with C24 carbons was very low for both cow as well buffalo ghee. It can be seen in (**Figure 4.1**) that small and medium carbon number TGs (C26 to C38 ) were higher for buffalo ghee than cow ghee.

For cow ghee C40 TG was higher than buffalo ghee. Higher carbon number TGs (C44-C54) was higher in case of cow ghee as compare to buffalo ghee. Earlier Kala (2013) also reported that higher carbon number TGs were higher in cow ghee as compare to buffalo ghee. It was also noted from **Figure 4.1** that TG with carbon number C50, C52 and C54 in case of buffalo ghee were significantly lower than cow ghee. So it was concluded that there was a difference in the concentration of (C44-C54) carbon number TGs in cow and buffalo ghee. Results of the present investigation are in the line with the findings of Kala, 2013 who reported that buffalo ghee contains less C52 and C54 TG than cow ghee. So, there might be a need to study the TGs profile of both these fats on pan India basis taking into the considerations of different regions and seasons to develop or validate the ISO/ IDF equations suggested for checking the quality of milk fat. Earlier Goudjil *et al.* (2003) also reported that C54 carbon number TG was an efficient biomarker to differentiate milk fat of different animal. The compositions and distribution of TG in milk exhibit many structural similarities for different animal species (Christie, 1989).TGs C38, C50 and C52 were observed maximum in cow milk fat reported by Smiddy *et al.* (2012). Smiddy *et al.* (2012) also reported that horse milk fat contained a greater percentage of C44 and donkey milk a greater percentage of C52. It is clear from above discussion that milk fat TG varies from species to species. This variation mainly depends on genetic variation as well as feeding habit. Fontecha *et al.* (1998) reported that TG content of milk fat not only varies on species to species but this variation could be observed in different breed of same species animal.

IDF & ISO (2010) had given five multiple regression equations to check the presence of different adulterant fats in milk fat. In the present study, the GLC conditions as specified by IDF & ISO (2010) could not be used due to some technical

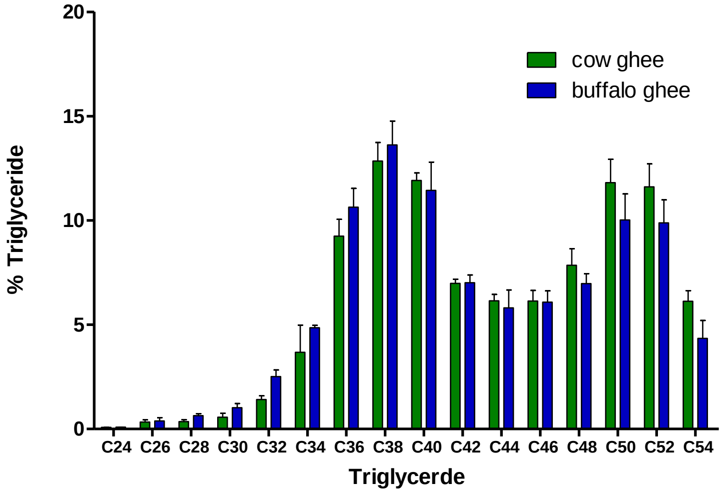


Figure 4.1: Comparison of Triglycerides profile of cow and buffalo ghee

problem as mentioned method was suitable for milk fat but in this study we used heat clarified milk fat i.e. ghee, so the actual validation of these equations in cow and buffalo ghee was not possible in true sense. However, the general equation i.e. (**$S_{total}$**) given by IDF & ISO for pure milk fat was tested.

**Table 4.18: $S_{total}$ values for cow and buffalo ghee**

| IDF & ISO (2010) equation | Recommended S value | S value for cow ghee | S value for buffalo ghee |
|---|---|---|---|
| -2.7575C26 + 6.4077C28 + 5.5437C30-15.3247C32 + 6.2600C34 + 8.0108C40-5.0336C42 + 0.6356C44 + 6.01171C46 | 95.68-104.32 | 107.0352 | 98.29 |

It is evident from **Table (4.18)** that in case of pure cow ghee S value was slightly exceeded from the range specified for milk fat (IDF & ISO 2010), but in case of buffalo ghee this value was within the range. This might have happened due to many reasons mentioning a few like different GLC conditions and method of extraction of milk fat as in the present case it was ghee. Fontecha *et al.* (1998) also reported that recommended S value cannot work in case of TG profiling of cheese due to biochemical reaction of cheese during ripening and storage. Kala (2013) also reported that S value of market sample of ghee ruled out recommended S value and this is due to the storage of ghee after preparation. In the present investigation also the ghee samples prepared were sealed in bottles and stored in a deep freezer before GLC analysis so that GLC analysis of all the samples could be performed once. This may have caused slight hydrolysis of the ghee which might have shifted the **$S_{Total}$** for cow ghee. Therefore, in the subsequent experiments the results were interpreted on the basis of high carbon number triglycerides instead of equations.

### 4.3.2 Triglycerides composition of some adulterant fats/ oils

In the present study lard, goat tallow and soy oil were used as adulterant fats.

#### 4.3.2.1 Triglycerides profile of lard

Lard is the animal body fat obtained from pig adipose tissue. Principal fatty acids found in lard are Oleic acid, Palmatic acid and stearic acid (Kim *et al.,* 2015). Fontecha *et al.* (1998) reported that fatty acid composition of fat directly influence the TG profile of fats and oil. In table **4.19** & (**Plate 4.32**) it is evident that short or smaller carbon number triglyceride were absent in lard whereas TG having C50,C52 & C54 carbons were the major TGs. In lard C52 TG was about 62.71±1.45 percent (w/w) and the major marker TG. Findings of the present study are in agreement with the findings of Kim *et al*. (2015) who also reported that C52 TG was in higher amount in lard.

#### 4.3.2.2 Triglycerides profile of goat body fat

Principal fatty acids found in goat body fat were Oleic acid, Palmatic acid and Stearic acid as reported by Upadhyay (2014). In the present study it was observed that TGs having carbon numbers C50, C52 & C54 were the major TGs (**Table4.19 and Plate 4.33)** in goat tallow.

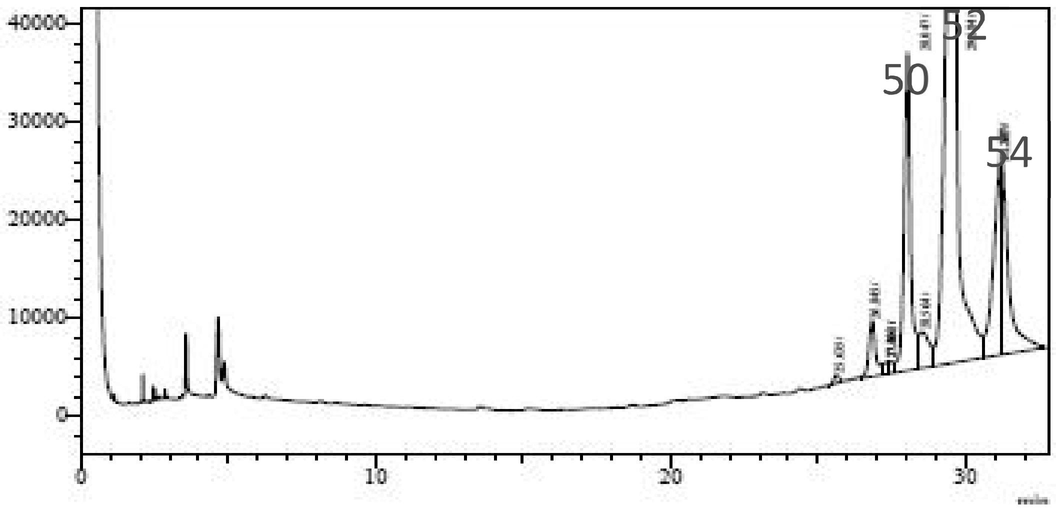


Plate 4.32: GLC Chromatogram of Lard Triglycerides profile

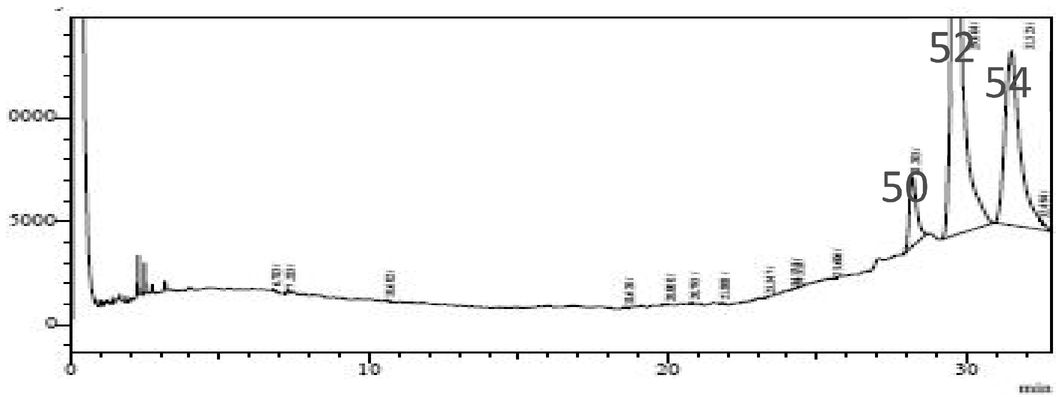


Plate 4.33: GLC Chromatogram of goat tallow Triglycerides profile

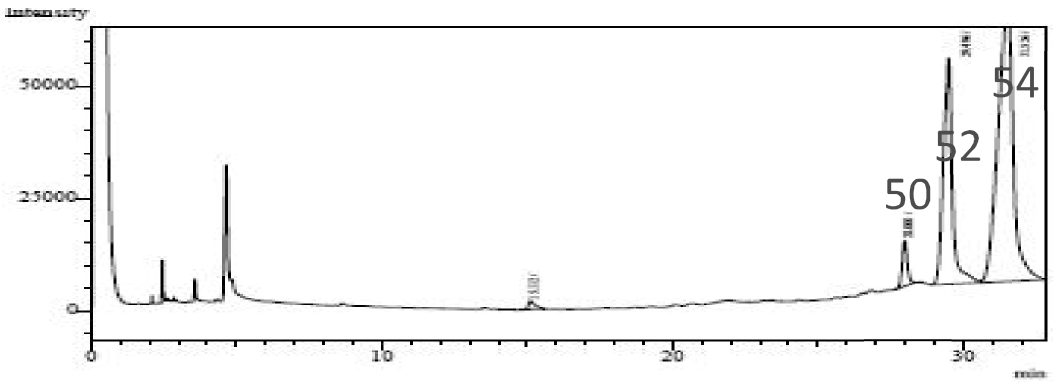


Plate 4.34: GLC Chromatogram of soy oil Triglycerides profile

**Table 4.19: Triglyceride (percent w/w) profile of some selected adulterant**

| TGs | Lard | Goat Body Fat | Soy oil |
|---|---|---|---|
| C24 | ND | ND | ND |
| C26 | ND | ND | ND |
| C28 | ND | ND | ND |
| C30 | ND | ND | ND |
| C32 | ND | ND | ND |
| C34 | ND | ND | ND |
| C36 | ND | ND | ND |
| C38 | ND | ND | ND |
| C40 | ND | ND | ND |
| C42 | ND | ND | ND |
| C44 | ND | ND | ND |
| C46 | ND | ND | ND |
| C48 | ND | ND | ND |
| C50 | 11.78±3.4 | 24.17±2.9 | 3.465±1.65 |
| C52 | 62.71±1.45 | 34.54±6.8 | 31.0845±5.8 |
| C54 | 25.51±1.63 | 26.99±5.9 | 64.0195±4.65 |

Data represents average value± SD value (n=6),ND-not detected

Similar type of observations were recorded by Kala (2013) in case of bovine tallow and reported that C50, C52 & C54 carbon number TGs were major TGs found in tallow. Kim *et al*. (2015) reported that animal body fat contains higher amount of C50, C52 & C54 and these TGs could be a potent marker to differentiate pure and milk fat adulterated with animal body fat.

### 4.3.2.3 Triglycerides profile of soy oil

In (**Plate 4.34**) and **table 4.19** it can be seen that unlike body fats, in case of soy oil the main TGs were C50, C52 & C54. Results of the present study are in line with earlier observation of Kim *et al.* (2015) who reported that soy oil has no C24-C48 carbon number TG and C54 was the main TG identified in soy oil.

As higher chain length fatty acid are higher in soy oil so higher carbon number TG were the major TG observed in soy oil. As compare to animal body fat soy oil had very high amount (64.0195±4.65 percent-w/w) of C54 TG (**Table 4.19**).

### 4.3.3 Triglycerides profile of ghee (cow and buffalo) adulterated with lard

Detection of animal body fat in milk fat has always been a challenge (Kumar *et al.*, 2009). It is evident from the results that on adulterating ghee (cow or buffalo) with lard, smaller carbon number TGs (C24 & C26) remained undetected (**Table 4.20**). This might have happened due the dilution effect as lard had no C24 to C48 TGs but very high TGs with carbon number C50, C52, and C54.

### Table 4.20: Triglycerides (percent w/w) profile of ghee adulterated with lard

| | Cow Ghee | | | | Buffalo Ghee | | | |
|---|---|---|---|---|---|---|---|---|
| **TG** | **Pure** | **20% Lard** | **10% Lard** | **5% Lard** | **Pure** | **20% lard** | **10% lard** | **5% lard** |
| **C24** | 0.075±0.001 | ND | ND | ND | 0.086±0.003 | ND | ND | ND |
| **C26** | 0.33±.11 | ND | ND | ND | 0.39±0.15 | ND | ND | ND |
| **C28** | 0.35±0.10 | 0.26±0.008 | 0.28±0.01 | 0.32±0.004 | 0.64±0.10 | 0.43±0.12 | 0.55±0.06 | 0.58±0.04 |
| **C30** | 0.57±0.19 | 0.22±0.01 | 0.26±0.14 | 0.26±0.19 | 1.02±0.20 | 0.39±0.05 | 0.46±0.11 | 0.63±0.90 |
| **C32** | 1.41±0.18 | 0.42±0.12 | 0.47±0.36 | 0.88±0.14 | 2.51±0.32 | 2.09±0.13 | 2.17±0.06 | 2.29±0.19 |
| **C34** | 3.68±1.30 | 1.094±0.15 | 1.11±0.36 | 2.1±0.40 | 4.85±0.12 | 2.96±0.12 | 3.42±0.36 | 4.56±0.11 |
| **C36** | 9.25±0.80 | 4.04±0.70 | 4.957±0.23 | 6.34±0.11 | 10.64±0.9 | 7.63±0.45 | 7.69±0.39 | 8.1±0.54 |
| **C38** | 12.85±0.89 | 7.96±0.11 | 8.21±0.35 | 10.20±0.34 | 13.62±1.14 | 7.28±0.11 | 8.65±0.36 | 9.04±0.16 |
| **C40** | 11.92±0.36 | 7.41±0.42 | 8.96±0.21 | 9.10±0.35 | 11.44±1.36 | 10.40±0.29 | 10.85±0.23 | 11.01±0.24 |
| **C42** | 6.98±0.20 | 4.69±0.23 | 4.89±0.33 | 6.54±0.23 | 7.017±0.38 | 4.67±0.34 | 4.93±0.43 | 5.32±0.21 |
| **C44** | 6.15±0.30 | 3.254±0.68 | 4.065±1.12 | 5.19±1.3 | 5.81±0.86 | 3.16±1.20 | 3.54±.0.63 | 4.55±0.35 |
| **C46** | 6.14±0.50 | 3.88±1.43 | 4.55±0.63 | 6.40±0.59 | 6.08±0.54 | 3.14±0.56 | 3.63±0.74 | 3.79±0.43 |
| **C48** | 7.85±0.80 | 4.34±1.20 | 4.82±1.67 | 5.89±0.54 | 6.97±0.48 | 4.89±0.32 | 5.32±0.3 | 5.72±0.16 |
| **C50** | 11.81±1.12 | 14.78±1.10 | 14.28±1.56 | 12.24±1.63 | 10.02±1.25 | 15.05±0.90 | 11.45±2.10 | 10.82±1.63 |
| **C52** | 11.61±1.30 | 28.68±1.40 | 19.40±1.64 | 15.06±1.84 | 9.88±1.10 | 21.65±1.23 | 17.86±1.7 | 14.44±1.54 |
| **C54** | 6.13±0.50 | 14.46±1.32 | 10.34±1.32 | 8.32±1.33 | 4.35±0.85 | 11.65±1.23 | 8.54±1.32 | 7.34±1.56 |

Data represents average value±SD value (n=6),ND-not detected

It was clearly visible from Fig (**4.2 & 4.3**) that adulterated sample of ghee had higher C50, C52 & C54 than pure ghee. A direct correlation was observed in the increase of C50, C52 & C54 with the level of adulteration of lard. It was also observed that small and medium carbon number TGs decreased (**Fig 4.2 & 4.3**). Kim *et al.* (2015) and Destaillats *et al.* (2006) reported that C54 TG alone could be efficiently used as an indicator to detect adulteration in milk fat. In the present study it was observed that lard had higher amount of C50, C52 & C54 than milk fat, so summation of these three TGs was found to be more useful approach to detect lard in ghee than individual TGs.

**Table 4.21: Summation of (C50+C52+C54) TGs for pure ghee (cow/buffalo) and ghee sample adulterated with lard**

| **Summation of TG (C50+C52+C54)** | **Cow ghee** | | | | **Buffalo ghee** | | | |
|---|---|---|---|---|---|---|---|---|
| | 0% lard | 20% lard | 10% lard | 5% lard | 0% lard | 20% lard | 10% lard | 5% lard |
| | 29.55±1.18 | 58.24±1.69 | 44.02±2.26 | 36.62±1.45 | 24.25±2.16 | 48.35±1.34 | 37.85±1.73 | 32.6±1.85 |

It is clearly visible from **Table (4.21)** that summation of three TGs (C50+C52+C54) had significantly higher value for ghee adulterated with lard as compare to the pure ghee. Using summation of the TGs (C50+C52+C54) adulteration of ghee with lard to the tune of minimum 5% could be detected.

### 4.3.4 Triglycerides profile of ghee (cow and buffalo) adulterated with goat tallow

In the case of ghee (cow or buffalo) samples adulterated with goat body fat it was observed that smaller carbon number TGs went missing in the chromatogram like earlier case wherein lard was used as an adulterant. This can again be attributed

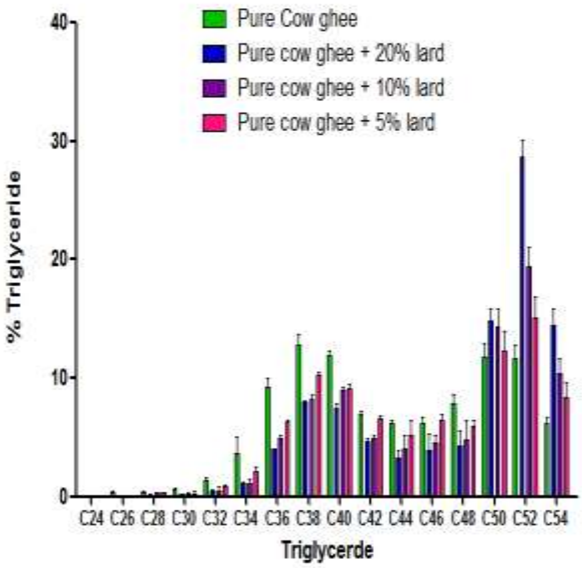


Figure 4.2: Triglycerides profile in cow ghee adulterated with lard

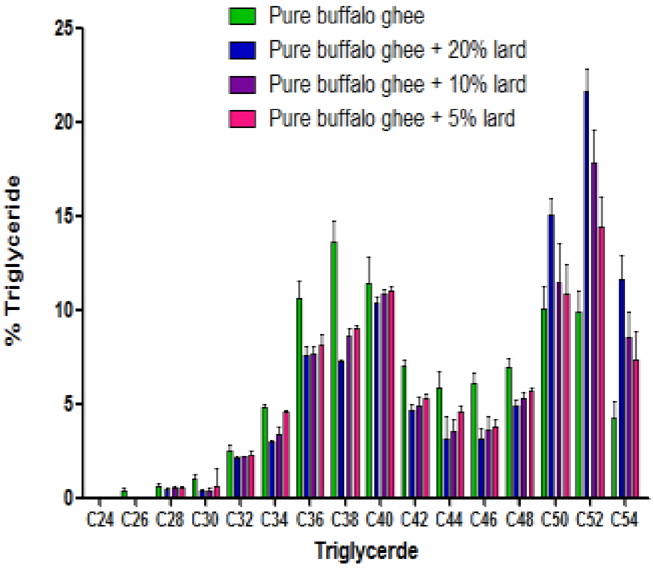


Figure 4.3: Triglycerides profile in buffalo ghee adulterated with lard

to the absence of low carbon number TGs in goat tallow. It is also evident from the results depicted in Table **4.22** that medium carbon number C36 to C48 TGs area and peak height also decreased. However, the area and peak height of TGs with higher carbon numbers i.e. C50 ,C52 and C54 increased (**Table 4.22 & Fig 4.4 and Fig 4.5)** and the increase was directly proportional to the level of adulteration. This was expected because goat body fat had no C24 to C48 TGs .The C50, C52, and C54 TGs in goat body fat were higher than those in pure ghee.

### Table 4.22 : Triglycerides ( per cent w/w) profile of ghee adulterated with goat body fat/Goat tallow (GT)

| | Cow Ghee | | | | Buffalo Ghee | | | |
|---|---|---|---|---|---|---|---|---|
| **TG** | **Pure** | **20% Goat body fat** | **10% Goat body fat** | **5% Goat body fat** | **Pure** | **20% Goat body fat** | **10% Goat body fat** | **5% Goat body fat** |
| **C24** | 0.075±0.001 | ND | ND | ND | 0.086±0.003 | ND | ND | ND |
| **C26** | 0.33±11 | ND | ND | ND | 0.39±0.15 | ND | ND | ND |
| **C28** | 0.35±0.10 | 0.23±0.09 | 0.33±0.12 | 0.56±0.33 | 0.64±0.1 | 0.33±0.12 | 0.59±0.36 | 0.69±0.82 |
| **C30** | 0.57±0.19 | 0.49±0.23 | .56±0.34 | .82±0.11 | 1.02±0.2 | 0.6±0.36 | 0.89±0.14 | 1.03±0.19 |
| **C32** | 1.41±0.18 | 1.09±0.11 | 1.55±0.32 | 1.67±0.36 | 2.51±0.32 | 1.02±0.14 | 1.05±0.16 | 1.32±0.14 |
| **C34** | 3.68±1.30 | 3.06±0.39 | 3.41±0.44 | 3.59±0.55 | 4.85±0.12 | 3.69±0.32 | 3.89±0.41 | 4.09±0.33 |
| **C36** | 9.25±0.80 | 8.84±1.36 | 8.89±1.44 | 9.1±1.39 | 10.64±0.9 | 9.54±1.63 | 9.89±.87 | 9.98±.53 |
| **C38** | 12.85±0.89 | 7.29±1.36 | 7.91±1.89 | 8.95±1.42 | 13.62±1.14 | 9.668±1.64 | 10.16±1.33 | 10.54±1.65 |
| **C40** | 11.92±0.36 | 11.49±1.54 | 11.82±1.54 | 11.90±1.73 | 11.44±1.36 | 9.69±..42 | 10.05±1.06 | 10.32±1.05 |
| **C42** | 6.98±0.20 | 5.87±1.54 | 6.12±1.32 | 6.76±1.54 | 7.017±0.38 | 5.69±1.04 | 5.80±0.9 | 5.85±1.42 |
| **C44** | 6.15±0.30 | 4.70±1.54 | 5.12±1.43 | 5.93±1.23 | 5.81±0.86 | 4.84±.34 | 5.02±.41 | 5.55±.34 |
| **C46** | 6.14±0.50 | 3.57±1.33 | 4.89±1.52 | 5.23±1.33 | 6.08±0.54 | 4.87±0.76 | 5.19±0.62 | 5.42±0.54 |
| **C48** | 7.85±0.80 | 6.27±0.12 | 5.87±0.43 | 6.04±0.11 | 6.97±0.48 | 5.87±0.32 | 6.01±0.22 | 6.26±0.24 |
| **C50** | 11.81±1.12 | 13.998±1.32 | 12.93±1.54 | 12.16±1.86 | 10.02±1.25 | 14.42±0.75 | 14.86±2.12 | 10.57±3.43 |
| **C52** | 11.61±1.30 | 24.32±2.21 | 22.27±1.68 | 19.2±1.25 | 9.88±1.10 | 17.4±1.34 | 15.86±1.65 | 13.57±1.32 |
| **C54** | 6.13±0.50 | 12.39±1.12 | 12.45±1.56 | 10.20±1.45 | 4.35±.85 | 11.2±1.39 | 9.55±1.23 | 8.689±.98 |

Data represents average value± SD value (n=6),ND-not detected

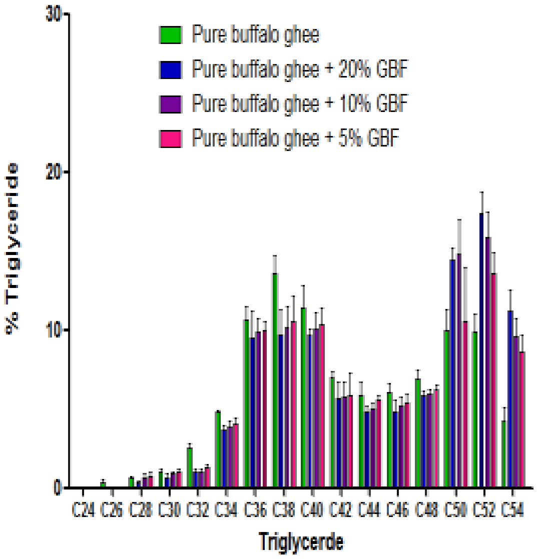


**Figure 4.4: Triglycerides profiling of buffalo ghee adulterated with goat body fat (GBF)/Goat tallow**

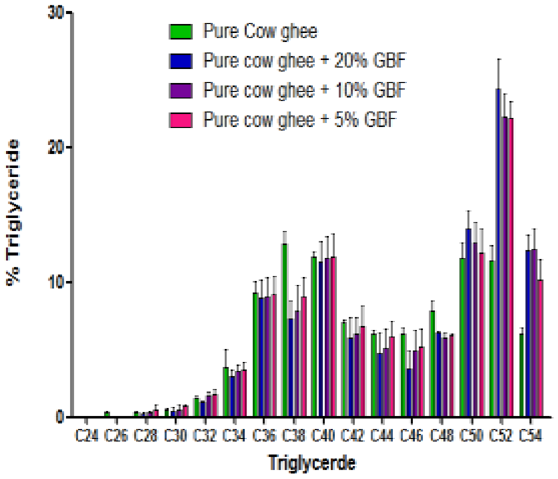


**Figure 4.5: Triglycerides profiling of cow ghee adulterated with goat body fat (GBF)/Goat tallow**

**Table 4.23: Summation of (C50+C52+C54) TGs for pure ghee (cow/buffalo) and ghee sample adulterated with goat tallow (GT)**

| Summation of TG (C50+C52+C54) | Cow ghee | | | | Buffalo ghee | | | |
|---|---|---|---|---|---|---|---|---|
| | 0% GT | 20% GT | 10% GT | 5% GT | 0% GT | 20% GT | 10% GT | 5% GT |
| | 29.55±1.18 | 51.70±2.34 | 47.65±2.56 | 41.01±1.45 | 24.25±2.16 | 43.02±3.16 | 39.27±1.45 | 32.82±1.78 |

On summing up the C50+C52+C54 TGs it was found that the percent concentration was significantly higher as compare to pure ghee (**Table 4.23**). Using this concept of summation, goat tallow added to ghee could be detected to the tune of minimum 5%.

### 4.3.5 Triglycerides profile of ghee (Cow and buffalo) adulterated with soy oil and lard

Lard is the animal body fat obtained from pig adipose tissue. Principal fatty acid found in lard is that Oleic acid, Palmatic acid and stearic acid. It is evident from the results discussed in section **4.3.2.1** that Small and medium carbon number triglyceride were absent in lard whereas TG C50, C52 & C54 were the major ones. The major TG identified in lard was C52 having 52 carbons. However, Oleic and Linoleic acids were the major fatty acids in soy oil. Therefore, in case of Soybean (soy) oil short and medium chain (C24-C48 carbon number) TGs were also absent, only C50, C52 & C54 TGs were predominant in soy oil and C54 carbon number TGs were the major TG identified in soy oil. So it was clear that for animal body fat (Lard) C52 carbon number TG and in case of soy oil C54 carbon number TG were the major TG.

**Table 4.24: TG (percent w/w) profile of cow and buffalo ghee adulterated with 5% soy oil + 5% lard**

| TG | Cow Ghee | Buffalo Ghee |
|---|---|---|
| | 5% Soy oil+5% Lard | 5% Soy oil+5% Lard |
| C24 | ND | ND |
| C26 | ND | ND |
| C28 | 0.14±0.12 | 0.12±0.09 |
| C30 | 0.19±0.16 | 0.89±0.06 |
| C32 | 0.79±0.11 | 0.77±0.13 |
| C34 | 0.58±0.12 | 0.59±0.14 |
| C36 | 7.62±0.19 | 8.06±0.35 |
| C38 | 11.48±1.23 | 11.88±0.95 |
| C40 | 10.78±0.8 | 10.38±0.68 |
| C42 | 6.77±0.54 | 6.54±0.43 |
| C44 | 5.63±0.11 | 5.92±0.87 |
| C46 | 6.31±0.35 | 6.48±0.45 |
| C48 | 8.66±0.57 | 8.78±1.01 |
| C50 | 14.52±0.87 | 14.235±0.96 |
| C52 | 21.45±0.15 | 17.62±0.32 |
| C54 | 11.89±0.11 | 8.91±1.46 |
| Data represents average value±SD value (n=6),ND-not detected | | |

It is evident from the data presented in table 4.24 that on addition of lard and soy oil @ 5% each in ghee, the short and medium size TGs were decreased and long carbon number TGs (C50,C52 & C54) were found to be increased. It is also evident from the results that TGs with C50, C52 & C54 had highest value in case of ghee adulterated with admixture of lard and soy oil than pure ghee as well as ghee adulterated with lard alone (**Fig. 4.6 & 4.7**). It was also seen, as summation value of TGs C50+C52+C54 had significantly higher value for ghee adulterated with admixture of lard + soy oil (@ 5%+5%) than ghee adulterated with lard (@5%) alone (Table 4.25). Hence, it can be concluded that there is a possibility to detect the adulteration of ghee with admixture of lard + soy oil to the tune of (5%+5%).

Upadhyay (2014) reported that fatty acid analysis of ghee adulterated with admixture of animal body fat and vegetable oil could not be a fruitful technique for detection of adulteration in ghee. But present investigation clearly demonstrated that

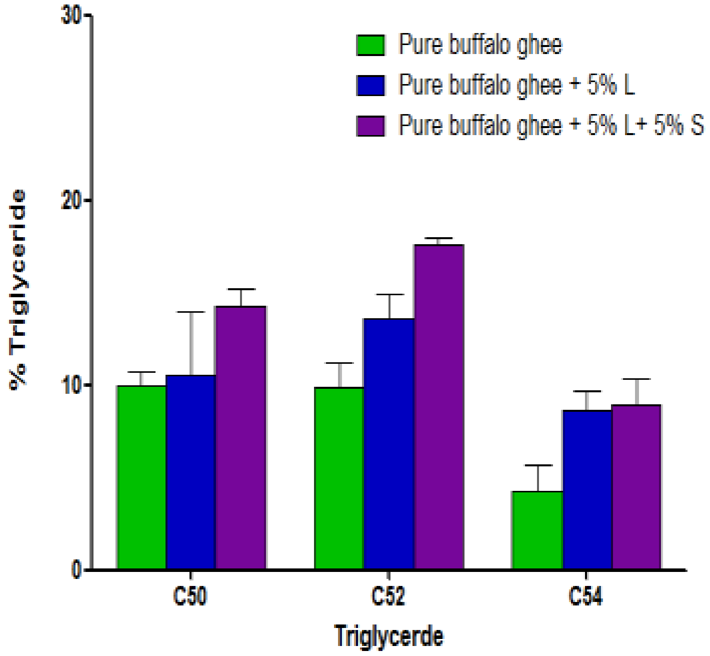


**Figure 4.6: Comparison of (C50,C52 & C54) TG profile of Pure buffalo ghee, buffalo ghee added with 5% lard & buffalo ghee added with 5% lard + 5% soy oil (s)**

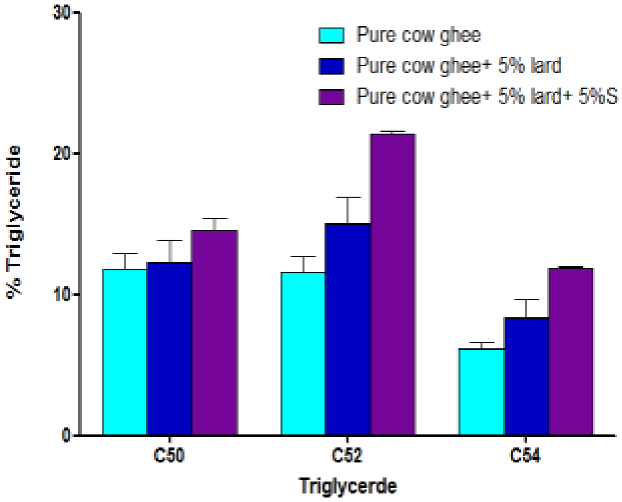


**Figure 4.7 : Comparison of** (C50,C52 & C54) TG profile of **Pure cow ghee, cow ghee added with 5% lard & cow ghee added with 5% lard + 5% soy oil (s)**

TG analysis and summation of TGs carbon number (C50+C52+C54) had significantly higher value than pure ghee (cow and buffalo ghee), that clearly differentiate pure ghee and adulterated ghee (admixture of lard + soy oil).

**Table 4.25:Summation of TGs (C50+C52+C54)cow and buffalo ghee adulterated with 5% soy oil + 5% lard**

| **Summation of TG (C50+C52+ C54)** | **Cow ghee** | | **Buffalo ghee** | |
|---|---|---|---|---|
| | Pure | 5% Lard+5% Soy oil | Pure | 5% Lard +5% Soy oil |
| | 29.55±1.18 | 47.86±2.39 | 24.25±2.16 | 40.76±2.86 |

### 4.3.6 TG profile of ghee (Cow and buffalo) added with soy oil and goat tallow (GT)

As in case of lard, small and medium carbon number triglycerides were absent in goat tallow too and TGs with carbon numbers 50, 52 & 54 were the major TGs. In case of Soybean (soy) oil as discussed in previous section, TGs with higher carbon numbers i.e. C50, C52 & C54 were predominant. As compared to animal body fat soy oil has higher amount of C54 TG.

**Table 4.26:TG (percent w/w) profile of cow and buffalo ghee adulterated with of 5% soy oil + 5% goat tallow (GT)**

| **TG** | **Cow Ghee** | **Buffalo Ghee** |
|---|---|---|
| | 5% Soy oil+5% GT | 5% Soy oil+5% GT |
| **C24** | ND | ND |
| **C26** | ND | ND |
| **C28** | 0.09±0.005 | 0.16±0.09 |
| **C30** | 0.24±0.16 | 0.49±0.18 |
| **C32** | 0.63±0.11 | 0.57±0.13 |
| **C34** | 0.44±0.12 | 0.69±0.14 |
| **C36** | 6.76±0.19 | 7.86±0.15 |
| **C38** | 11.28±1.23 | 11.54±0.95 |

| | | |
|---|---|---|
| **C40** | 9.0±0.8 | 11.51±0.68 |
| **C42** | 6.57±0.54 | 6.11±0.43 |
| **C44** | 5.83±0.11 | 6.65±0.87 |
| **C46** | 6.43±0.35 | 6.28±0.65 |
| **C48** | 8.78±0.57 | 8.48±1.01 |
| **C50** | 16.76±0.87 | 13.235±0.56 |
| **C52** | 18.45±0.35 | 18..62±0.32 |
| **C54** | 12.45±2.35 | 11.33±2.46 |
| Data represents average value±SD value (n=6) | | |

It is evident from the results depicted in **table 4.26** that when goat tallow and soy oil were added @5%+5% each in ghee, small and medium carbon number TGs were decreased and long carbon number TGs (C50,C52 & C54) were increased. This is attributed to the fact that both goat tallow as well as soy oil contain higher carbon number (C50, C52 & C54) TGs in higher percentage than that of pure milk fat. It is evident from the data that C50, C52 & C54 had highest value in case of ghee adulterated with admixture of goat body fat and soy oil, than pure ghee. It was also observed that on adding goat tallow and soy oil both at a time in ghee, long chain TGs (C50,C52 & C54) found to increase more in comparison to ghee adulterated with goat tallow alone at @ 5% ( **Fig. 4.8 & 4.9**).

Summation data of these three TGs clearly indicated presence or absence of adulterants in ghee. Data shown in table (4.27) clearly indicated that summation of three TGs (C50,C52 & C54) had significantly higher value for ghee adulterated with the admixture of soy oil + GT (@5%+5% level) than pure ghee.

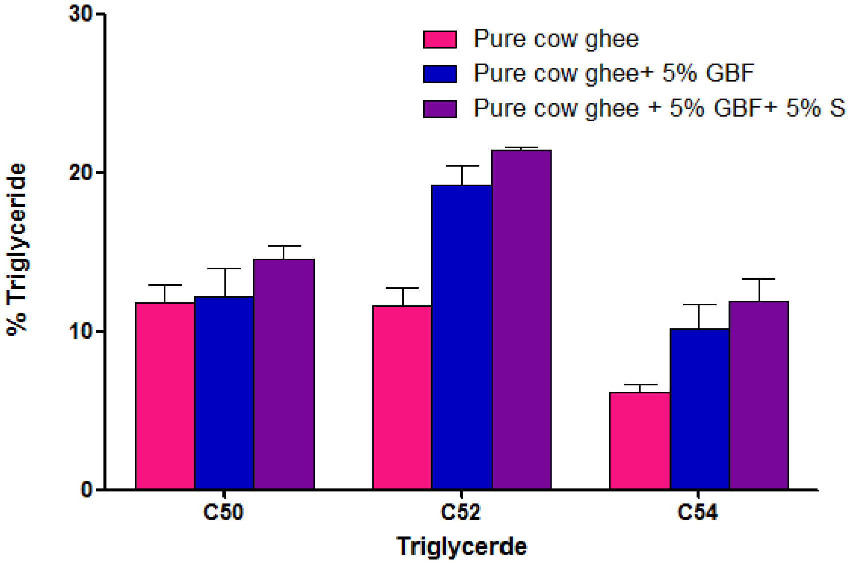


Figure 4.8: Comparison of (C50,C52 & C54) TG profile of Pure Cow ghee, Cow ghee added with 5% Goat tallow (GBF) & Cow ghee added with 5% Goat tallow (GBF/GT)+ 5% soy oil (s)

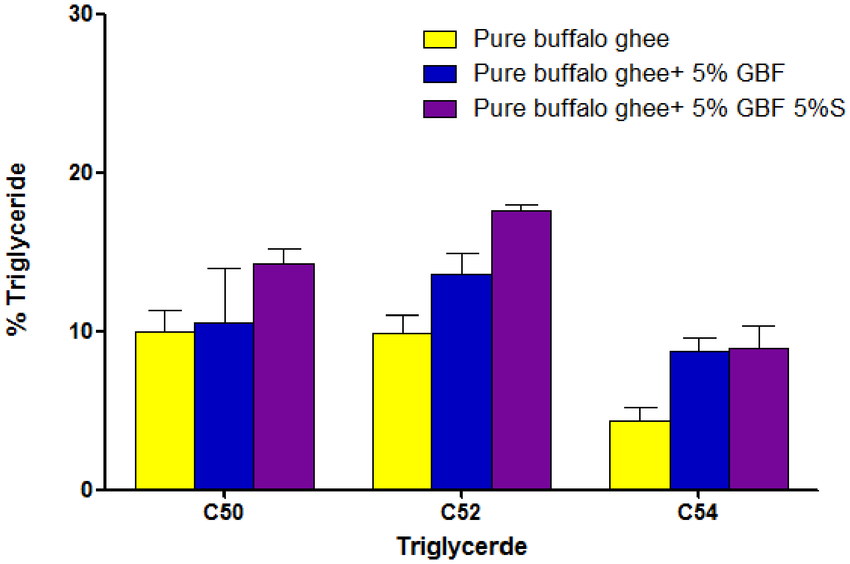


Figure 4.9 : Comparison of (C50,C52 & C54) TG profile of Pure buffalo ghee, buffalo ghee added with 5% Goat tallow (GBF/GT)& buffalo ghee added with 5% Goat tallow (GBF/GT)+ 5% soy oil (s)

**Table 4.27:Summation of TGs (C50+C52+C54)cow and buffalo ghee adulterated with 5% soy oil + 5% goat tallow (GT)**

| Summation of TG (C50+C52+ C54) | Cow ghee | | Buffalo ghee | |
|---|---|---|---|---|
| | Pure | 5% GT+5% Soy oil | Pure | 5% GT+5% Soy oil |
| | 29.55±1.18 | 47.66±1.46 | 24.25±2.16 | 43.18±2.01 |

Patel (2011) reported, that detection of animal body fat as well as admixture of animal body fat with vegetable oil, in ghee using physico chemical constant analysis could not be fruitful technique. Upadhyay (2014) reported that TLC based techniques as well as fatty acid analysis of ghee using GLC not able to detect adulteration of ghee with admixture of animal body fat and vegetable oil. Sushil (2015) reported that FT-IR based study could not differentiate adulterated ghee (with body fat) from pure ghee. Hence, from above discussion it was clear that different traditional and modern analytical techniques failed to detect adulteration of ghee with body fat specially when admixture of body fat and vegetable oil was used as adulterant fat. However, analysis of TGs and summation variation C50, C52 & C54 was able to detect admixture of lard/GT + soy oil to the tune of 5%, but this technique could not confirm type of adulterant (vegetable oil/body fat/ or admixture of vegetable oil+ body fat) present in ghee. So, it can be concluded that it is possible to detect the adulteration of ghee with heat clarified animal body fats (goat tallow or lard) when used in conjunction with soy oil. However, it is still not possible to ascertain the type of heat clarified animal body fats added to ghee, when admixed with vegetable oils.

# CHAPTER - 5

# Summary and Conclusions

# 5. SUMMARY & CONCLUSION

A systemic study was conducted to ascertain the purity of ghee on the basis of genomic DNA analysis and triglyceride profiling using GLC. Cow and buffalo milk, were used for the preparation of respective ghee samples, which were procured from the Livestock Research Centre, NDRI, Karnal. Goat tallow, lard and soy oil were used as adulterant. Goat adipose tissue and pig adipose tissue was collected from local slaughter houses, were heat clarified to get body fat (lard and goat tallow). Soya (soy oil) oil was purchased from local market of Karnal. Pure ghee samples were heated to 60-70°C for 10 min before adding and mixing of adulterants.

**1-** Various traditional methods and commercial kits of DNA isolation were tried and evaluated for isolation of DNA from fat samples,that was suitable for subsequent downstream application. Purity and yield of DNA was checked by spectrophotometric analysis, quality of DNA was checked by 0.8% agarose gel electrophoresis. Amplification of DNA was checked by universal primer 1 targeting *mt-cytb* gene resulting into a product of 360 bp, for confirming the presence of DNA in fat samples. Another primer namely universal primer 2, targeting *mt-12srRNA* gene resulting into amplicon of 440 bp was also used to further confirm the efficiency of methods of DNA isolation.

**2-** It was observed from spectrophotometric data that for **Phenol chloroform based method of DNA isolation,** yield and purity of DNA was very poor and there was no DNA band visible on 0.8% agarose gel electrophoresis. No amplification was recorded by Universal primer 1. Hence these observations clearly confirmed the absence of DNA. Thus, it was concluded that Phenol chloroform based method was not able to isolate DNA from fat samples.

**3-** It was observed from spectrophotometric data that for **Phenol chloroform and isoamyl-alchol based method of DNA isolation,** yield and purity of DNA was very poor in this case also and there was no DNA band visible on 0.8% agarose gel. No amplification recorded by universal primer 1. Hence these observations clearly confirmed the absence of DNA.

**4-** It was observed from spectrophotometric data that for **Phenol chloroform and CTAB based method of DNA isolation,** yield and purity of DNA was again very poor and there was no DNA band visible on 0.8% agarose gel. No amplification recorded by universal primer 1. Hence these observations clearly confirmed the absence of DNA and thereby indicating that Phenol chloroform and CTAB based method was also not a candidate method for isolation of DNA from fat samples.

**5-** It was observed from spectrophotometric data that for **PBS followed by Nucleo Spin-tissue kit based method of DNA isolation** yield and purity of DNA was again very poor and there was no DNA band visible on 0.8% agarose gel electrophoresis. No amplification recorded by Universal primer 1. As PBS not able to penetrate fat matrices and thus no DNA was released from fat samples hence it was not possible to isolate DNA using PBS followed Nucleo Spin tissue kit based protocol.

**6-** It was observed from spectrophotometric data that **water extraction followed by Nucleo Spin tissue kit and DNeasy mericon food kit based methods of DNA isolation,** yield and purity of DNA was very poor and there was no DNA band visible on 0.8% agarose gel electrophoresis. No amplification recorded by universal primer 1. This may be ascribed to the fact that water used in this protocol might not be able to penetrate fat matrix to liberate DNA. Hence this protocol was also not found suitable for isolation of DNA from fat samples. Same observation was recorded for DNA isolation from fat samples using water extraction method followed by using Dneasy Mericon food kit isolation protocol. Hence those protocols were also not suitable for isolation of DNA from fat samples.

**7-** Spectrophotometric data clearly revealed that DNA could be isolated by **Dneasy Mericon food kit based method** but there was no DNA visible on 0.8% gel and no amplification was recorded by Universal primer 1. This clearly confirmed that using this method it could be possible to isolate DNA but protocol might not be able to purify DNA from PCR inhibitor that stall the *Taq polymerase* activity, hence no amplification was recorded. So this protocol was also found to be unsuitable for DNA isolation from fat samples.

**8-** Spectrophotometric data clearly revealed that **modified NucleoSpin-tissue kit based method** could able to isolate DNA but yield and purity of isolated DNA were very low, also it was observed that no DNA was visible on 0.8% agarose gel. A very faint band of 360 bp amplicon was visible from DNA sample after amplifying with universal primer 1,so these result clearly confirmed that modified Nucleo Spin tissue kit based method was able to isolate DNA from fat samples. Since the isolated DNA seemed to be highly degraded or diluted, hence it was concentrated by mini concentrator. After concentration it was also found that yield and purity of DNA isolated from fat samples was better. But, in this case also no DNA band was visible on 0.8% gel electrophoresis. However a sharp band of 360 bp amplicon was visible on 2% agarose gel electrophoresis that clearly confirmed the presence of DNA and amplification of universal primer 1. To reconfirm the presence of DNA another primer i.e. universal primer 2 yielding 440 bp amplicon was also tested. It was noted that DNA isolated from fat samples using modified NucleoSpin tissue kit based method followed by concentration of eluted DNA, could be amplified by universal primer 2. So, these results clearly confirmed that the protocol was able to isolate DNA from fat samples but this said method needed another instrument to concentrate DNA. To further peruse a simplified method, another kit i.e. QIAamp stool kit based protocol was evaluated to isolate DNA from fat samples.

**9-** Spectrophotometric data clearly revealed that QIAamp stool kit yield and purity of DNA were increased from previous method. In this case also DNA presence in the eluted material could not be confirmed by simple 0.8% gel electrophoresis. However, a sharp band of 360 bp as well as 440 bp amplicon were formed as a result of amplification of eluted DNA and amplified DNA band were visible on 2% agarose gel.

So, was found to be a suitable approach to isolate DNA of sufficient quantity and quality from ghee and heat clarified animal body fat (HCABF), which can be used for the detection of species specific fat in ghee.

**10-** After standardization of efficient protocol of DNA isolation from fat samples, a species specific PCR based methodology was standardized to detect goat tallow and lard adulteration in ghee.

**11-** Four sets of goat specific primers (T1, T2, T3 and T4) as reported in literature were tested in goat DNA isolated from goat blood. Among those four sets of primers only T4 primer was able to amplify goat DNA from goat blood resulting into the formation of 294 bp amplicon, but T4 primer had a cross amplification with buffalo species. To make it more specific concentration of $MgCl_2$ was standardized wherein it was found to be 3.5mM which made this primer highly specific for goat. Primer T4 after following the standardized conditions of PCR was found to be able to amplify DNA isolated from goat tallow using modified NucleoSpin tissue kit based protocol as well as QIAamp stool kit based protocol. It was observed that in both the cases 294 bp amplicon was visible on 2% agarose gel. On applying the standardized protocols of DNA isolation and PCR conditions in ghee samples adulterated with goat tallow it was found that the level of detection in case of NucleoSpin tissue kit based protocol was to the tune of 20% and level of detection for QIAamp stool kit based protocol followed by goat specific primer PCR was to the tune of minimum 10%. In case of ghee adulterated with admixture of vegetable oil and goat body fat the level of goat tallow detection remained the same i.e. minimum 10%. As level of detection for QIAamp stool kit based protocol was better than the NucleoSpin tissue kit based protocol, so to detect lard in ghee only QIAamp stool kit based protocol followed by pig specific PCR approach was used.

**12-** Five sets of pig specific primers (L1, L2, L3,L4 and L5) as reported in literature were selected to amplify pig DNA isolated from pig blood. Among these five sets of primers only L3 & L5 primer were found to be able to amplify pig DNA from pig blood resulting into a 712 bp amplicon and 149 bp amplicon respectively. Both the sets of primer were specific to pig DNA and had no cross amplification with cow or buffalo DNA. The same sets of primers were then tested in DNA isolated from lard by QIAamp stool kit protocol. It was observed that L3 primer did not work in the DNA isolated from lard. However, sharp band of 149 bp amplicon was observed in case of DNA isolated from lard confirming the efficient working of L5 primer in pig DNA isolated from lard. On using the selected primer L5 in ghee samples the detection of lard addition in ghee to the tune of minimum 10% was possible and even in the presence of vegetable oils in an admixture of vegetable oil and lard detection level remained the same and specific to lard.

**13-** Triglyceride (TG) profile of fat samples was obtained in a short length (2.5m) capillary column and using gas chromatograph (Shimadzu GC 2010 PLUS), equipped with flame ionization detector and temperature control module. A programmed of total 32.87 min was standardized. The 1µl sample (concentration 5mg/ ml) was injected at an initial temperature of 80°C maintained for 0.50 min and then raised temperature with a ramp rate of 50°C/min to 190°C by holding at this temperature for 1 min, then again raised up to 335°C with ramp rate 6°C/min and held at that temperature for 6min. Split ratio was set to 1:50.

**14-** There were 16 major peaks of even carbon number TGs , corresponding to TGs of 24 to 54 carbon number were observed in both cow or buffalo ghee. In both cow as well as buffalo ghee six TGs having carbon numbers (C36,C38,C40,C42,C50 & C52) were predominant representing about sixty percentage of total TGs present in both cow and buffalo ghee. Presence of C24 carbon number TG was almost negligible in cow or buffalo ghee. Smaller carbon number TG like (C26, C28, C30, C32) found to be very nominal, whereas TGs with carbon number C34- C54 were the major TGs in cow ghee or buffalo ghee. It was observed that triglyceride having carbon number 26 to 38, were more in buffalo ghee than cow ghee. In case of cow ghee triglyceride having 40 carbon was higher than buffalo ghee. It was also observed that triglyceride having carbon number 44 to 54 were higher in cow ghee than buffalo ghee.

**15-** In case of pure cow ghee $S_{total}$ value exceeded from the range specified for milk fat (IDF & ISO 2010), but in case of buffalo ghee this value was within the range specified by IDF & ISO (2010). Therefore $S_{total}$ such as specified in IDF & ISO (2010) is not applicable as such in ghee. Hence new specific equation i.e. summation equation found to be more useful in this case.

**16-** All three types of fats namely lard, goat tallow and vegetable oil (soybean oil) did not show the presences of TGs with C24 - C48 carbon numbers. In all these fats TGs having carbon numbers C50, C52 & C54 were observed. In case of animal body fat (lard and goat body fat) C52 TGs were major TG, whereas in case of soybean oil C54 TG was the major one. It was also noted that TGs with carbon number C50 was lowest among three (C50, C52 & C54) TGs found in both goat tallow, lard and soybean oil.

**17-** It was also observed that in ghee samples adulterated with lard the concentration of small and medium carbon number TGs was decreased and C24 & C26 TG were not identified in adulterated sample of ghee. It was also observed that higher carbon number TGs like (C50, C52 & C54) increased significantly as compare to pure ghee. Summation value of these three TGs was also significantly high in adulterated samples as compared to the summation value in pure ghee. On considering the summation value as marker the minimum level of detection of lard in ghee was to the tune of 5%.

**18-** In case of ghee adulterated with goat tallow a similar trend as that of ghee adulterated with lard was observed. In this case also small and medium carbon number TGs were decreased and C24 & C26 TG could not be identified in adulterated ghee sample. It was also observed that higher carbon number TGs like (C50, C52 & C54) increased significantly in adulterated samples as compare to pure ghee and summation value of these three TGs was also significantly high. The minimum level of detection of Goat tallow was also to the tune of 5%.

**19-** Present study revealed that in case of both heat clarified animal body fats and vegetable oil, longer carbon number TGs like (C50, C52 & C54) were higher than pure ghee. So whenever admixture of soybean oil and heat clarified animal body fats was used as an adulterant then the longer chain TGs like (C50, C52 & C54) increased significantly as in case of ghee adulterated with heat clarified animal body fats and vegetable oil individually. Therefore, it can be concluded that TGs profiling can be a useful tool to detect the adulteration of ghee with foreign fats but difficult to ascertain the type of fat (heat clarified animal body fats, vegetable oil or admixture) added to ghee.

From the results it was concluded that the PCR based methodology standardized in the present study was more specific and efficient to confirm the presence of lard and goat tallow in ghee even in the presence of adulterant vegetable oils. The minimum level of detection was found to be to the tune of 10%. Similarly, another method i.e. low- resolution GLC was also helpful in ascertaining the quality of ghee but not able to identify the type of adulterant fat. In this method the minimum level of detection was to the tune of 5%. The equations specified in the ISO/ IDF methods could not be used as such in ghee. This may be due to the variability in the make of column, slightly different conditions of GLC and type of sample i.e. ghee, instead of milk fat isolated by the methods specified in ISO/ IDF method.

Study also revealed that for Indian situation there is a need to map the TGs profile of milk fat available in the country considering the diversity in the milched animals and seasonal variations. There is also a need to develop new equations for the milk fat isolated from different dairy products available in the country.

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