MEASUREMENT OF CHOLESTEROL AND TRIGLYCERIDES IN FRESH SERUM AND DRIED SERUM AFTER STORAGE AT DIFFERENT TIME INTERVALS.

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KLE UNIVERSITY BELGAUM,
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LIST OF ABBREVIATIONS USED

DBS	-	Dried blood spots
NCD	-	Non communicable diseases
CVD	-	Cardiovascular diseases
MI	-	Myocardial infarction
LDL	-	Low density lipoprotein
US Hospital	-	United States Hospital
HIV	-	Human Immunodeficiency Virus
CRP	-	C Reactive Protein
EBV	-	Epstein Barr virus

ABSTRACT

Background and Objectives:

Non-communicable diseases (NCD) including cardiovascular diseases, diabetes, obesity, cancers, and chronic lung diseases, are growing concerns in developing countries. In a large country such as India, screening for NCD at remote corners of the country is difficult because of limited resources and technical capacity. Measurement in a good quality central laboratory would be ideal, but the cost and safety of chilled sample transportation are concerns. Transportation of samples in the form of dried blood/serum would circumvent the need for blood processing, storage, and shipment at ultralow temperatures. The collection of dried blood/serum spots on filter paper offers a powerful tool in screening programs. Dried serum spot technology offers several advantages over conventional serum assays; as it does not require separation of serum by centrifugation and allows convenient shipment of samples at low cost. Serum spot assays therefore allow a quick and convenient assessment of patients presenting cardiometabolic health risks. . In the present study, the stability of cholesterol and triglycerides in serum dried on filter paper at room temperature for different time intervals is studied.

Materials and methods:

100 Samples of Patients coming to the lab for lipid investigations were selected. Blood collected by venipuncture into tubes without anticoagulant was used. Replicates of serum were spotted onto 3M Whatman paper and left at room temp for an hour for drying. Filter discs were transferred to a plastic bag, sealed and stored at room temp for different time periods . Dried blood spots were cut out with scissors

and analysed on 7, 14, 21, 28 and 35 days in a Erba semiautoanalyser using commercially available kit.

Results

Cholesterol values in the 100 samples analyzed ranged from 102 mg/dl to 314 mg/dl.. The mean +/- standard deviation (SD) cholesterol values obtained from fresh serum was 148.33+/-30.68 mg/dl and the mean cholesterol values from corresponding dried serum was 147.86+/-30.67 mg/dl on the same day of drying and subsequently 147.59+/-30.47 (day 7) , 147.3+/-30.52 (day14) , 146.74+/-30.62 (day 21) , 146.45+/-30.69 (day 28) and 146.41+/-30.66 (day 35).

Triglyceride values in the 100 samples ranged from 86 mg/dl to 168 mg/dl. The mean +/- standard deviation (SD) triglyceride values obtained from fresh serum was 113.18 + 18.77 mg/dl and the mean cholesterol values from corresponding dried serum was 112.78 + 18.62 mg/dl on the same day of drying and subsequently 112.52 + 18.63 (day 7) , 112.35 + 18.64 (day14) , 111.87 + 18.70 (day 21) , 111.63 + 18.72 (day 28) and 111.49 + 18.80 (day 35).

A Intra class correlation coefficient of 0.98 for cholesterol and 0.99 for triglycerides was evident between dried serum spots and fresh serum.

Bland–Altman plots suggest that the difference in values obtained by the two methods were within the 2 SD limits for most of the samples for Cholesterol and for Triglycerides. Less than 5% of the values were outside the 2 SD limits.

Interpretation and conclusion:

The comparable values between dried serum spots and serum assays supports the usage of dried blood spot sample collection method as an alternative when conventional venous blood draw facilities are not available or accessible. However, precision and accuracy of the results can be improved by opting standard spotting methods and proper storage. The stability, efficient recovery, and excellent correlation with fresh serum samples makes the dried blood spot assay reliable and convenient method for screening modifiable risk factors of CVD like cholesterol and Triglycerides.

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INTRODUCTION

Guthrie and Susi introduced the concept of obtaining blood on filter paper in 1963 for the screening of metabolic diseases and since then the measurement of numerous analytes using the blood spot method have been published. The collection of Dried blood spots (DBS) in population-based research is a feasible and viable alternative to venous blood draws. The relative ease of sample collection, transport, and storage are significant benefits.[1]

In general, any analyte that can be measured from whole blood, serum or plasma can be measured from dried blood on filter paper. The dried blood/serum matrix stabilizes many analytes, including DNA, thereby allowing measurement of both phenotype (biochemical marker) and genotype (mutation or polymorphism) from a small volume of blood. Cellular components rupture when whole blood samples are dried on filter paper, which subsequently get released into solutions when they are reconstituted. Additional extraction procedures may be required for certain analytes to overcome this problem. Efficiency of elution of the analyte of interest and relative volume of sample collected are two other issues of concern in analysis with dried blood. Despite these limitations, dried blood/serum spots have several advantages. [2]

The filter paper blood collection device has achieved the same level of precision and reproducibility as that of standard methods for collecting blood, such as vacuum tubes and capillary pipettes.[3]

The advantages of Dried serum spot technology over conventional serum assays are , It is less invasive, and allows for convenient shipment of samples. Drying of serum also destroys infectious viruses, such as human immunodeficiency virus, and

ensures sample stability for weeks at room temperature, allowing for shipment without a cold pack or special precautions.[4.]

Screening for risk factors for non communicable diseases (NCD) in a country is essential to reduce the burden of these diseases in the population. The World Health Organization has recommended a three-step approach for NCD risk factor surveillance: (1) gather information through questionnaires (2) ascertain simple physical measurements and (3) collect blood sample.[5]

Due to complexities of the measurement involved, biochemical analysis is not done in all developing countries. Especially in a large country such as India, biochemical analysis at remote corners of the country will be difficult because of limited availability of resources and technical capacity. Carrying out the analysis in a good quality central laboratory is ideal, but the cost and safety of chilled sample transportation are concerns. Transportation of samples in the form of dried blood/serum would circumvent the need for blood processing, storage, and shipment at ultralow temperatures. [6]

Total cholesterol and triglycerides have been recognized as important modifiable risk factors for cardiovascular diseases. [7,8,9]

Recent studies support emergence of the term “cardiometabolic risk” defined by Watson as “the cluster of modifiable risk factors and markers that identify individuals at increased risk for cardiovascular disease (myocardial infarction, stroke, peripheral arterial disease) and type 2 diabetes.”[10]

Dyslipidaemias are one among the major modifiable risk factors for coronary heart disease.[11] Globally, 80% of deaths due to cardiovascular diseases occur in low and middle income countries.[12] According to the World Health Organisation (WHO) by 2030 more than 23 million people will die annually from CVD.[13] The primary and secondary intervention in developed countries have made CVD a disease of older age, whereas in developing countries the age of onset is at a younger age.[14]

A potential obstacle to the measurement of biomarkers in large epidemiologic, community based studies is the requirement for venous blood. Venipuncture is a relatively invasive procedure that must be performed by a trained phlebotomist (usually in a clinical setting), and it requires readily accessible facilities where blood samples can be promptly processed and stored under controlled conditions. Assays using whole blood dried on filter paper may provide a viable alternative.[15]

In the previous survellience studies, The following shortcomings were noted in the collection of blood/serum spots: spots were too small in some instances and disks could not be punched out for extraction. Spotting was done twice at the same spot in some samples. Filters were not dried properly prior to putting inside the resealable bags. The Ziploc bags were not closed tightly; as a result, moisture entered inside. Filter papers were not labeled properly. Because of the aforementioned factors, the variation in dried blood measurement was high, which is likely to affect the value of the assay in screening. For the dried blood approach to be successful in field conditions, variations in measurements due to these factors would need to be minimized.[16]

In the present study we assessed the stability of cholesterol and triglycerides in dried blood spots for 35 days and thereby validate the use of dried serum spots for screening cholesterol and triglyceride levels which will be helpful in providing information for early intervention and treatment for the individuals at increased risk for cardiovascular diseases.

AIMS AND OBJECTIVES

Aim:

Aim of the present study was to look for the stability of Cholesterol and Triglycerides in dried serum spots.

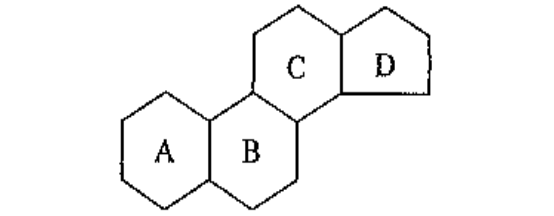
Objectives:

1. Comparing fresh serum Cholesterol and triglyceride levels with the levels of same in dried serum spots.
2. Evaluate the possibility of using dried serum spots instead of fresh liquid samples for screening the modifiable risk factors for cardiovascular diseases i.e Cholesterol and triglycerides.

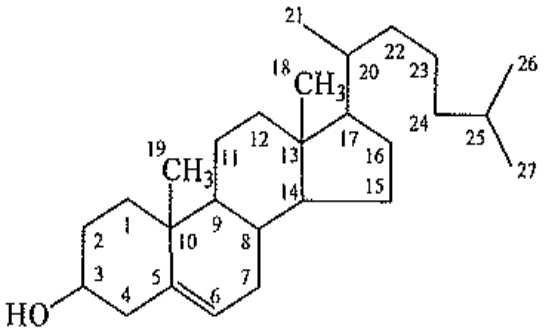
REVIEW OF LITERATURE

Structure of Cholesterol[16]

Cholesterol is found almost exclusively in animals and is a key membrane component of all cells. It is a steroid alcohol with 27 carbon atoms that are arranged in a tetracyclical sterane ring system,with a C-H side chain. Because of C-H bonds it is fairly water soluble . It does however contain a polar hydroxyl (OH) group on its A ring. Thus, it is both a polar and non polar molecule (Amphipathic)



Perhydrocyclopentanophenanthrene
(sterane) skeleton



Cholesterol

Figure Structure of cholesterol.

Triglycerides[16]

It is a Glycerol ester (Acylglycerol). The class of acylglycerol is determined by the number of fatty acyl groups present

1. One fatty acid – monoglycerides
2. Two fatty acid – diglycerides
3. Three fatty acid – triglycerides

Prevalence of Coronary Artery Diseases (CAD) increases with an increase in total cholesterol and triglycerides. The prevalence of CAD is rising rapidly in urban India. Lifestyle changes and aggressive control of risk factors are urgently needed to reverse this trend.[17]

Coronary artery disease remains a major public health problem in India as many Indians experience acute myocardial infarction (MI). India has 29.8 million coronary artery disease patients[18],1.27 million acute coronary artery disease patients and 11,13,359 undergo coronary angioplasty per year[19].

Asian Indians with CAD have similar levels of cholesterol as compared with white subjects but greater triglyceride levels. Triglycerides bring about a change in LDL particle size, density, distribution, and composition, producing smaller, denser, and more atherogenic particles. Both cholesterol and triglycerides, being modifiable risk factors, are amenable to change through public health and clinical interventions and therefore warrant early detection at the individual level and surveillance at the population level.[20]

The importance of cholesterol and triglycerides in Indian population is however, being reassessed in large samples by multicentric studies. These multicentric studies create special challenges with regards to laboratory

investigations. Choices are between performing the investigations at each of the centre or alternatively at the central laboratory. Carrying out investigations at each centre is likely to result in lot of variability. The later choice of a central laboratory performing the investigations is beset with problems of transportation. There are often problems like leakage and spillage of samples. Often a delay of several days occur before samples reach the laboratory. A suitable transport system is therefore a prerequisite in analysis at a central laboratory.

Dr. Robert Guthrie first began collecting heel-prick, blood spot samples from newborns to detect phenylketonuria . This effort has led to the implementation of a nationwide screening program in which DBS samples are collected from all newborns and then evaluated for a number of treatable metabolic disorders "Guthrie papers" have been a core component of US hospital-based newborn-screening programs since the 1960s and are subject to a rigorous quality-control program [21]

The filter paper matrix stabilizes most analytes in dried blood spots, but the rate of sample degradation will vary by analyte. Stability should be evaluated prior to sample collection because this has direct implications for sample handling and storage. For example, antibodies against the Epstein-Barr virus (an indirect measure of cell- mediated immunity) are stable in blood spots stored at room temperature for at least 8 weeks .[22] However, samples begin to deteriorate after 1 week of storage at 37°C. In contrast, concentrations of C-reactive protein decline significantly in DBS after three days at 37°C but are stable for at least 2 weeks at room temperature (20–23°C) [8]

Although refrigerating or freezing samples promptly after drying is always advisable to minimize the chances of degradation, the stability of most analytes in

DBS provides flexibility in the collection of samples in field settings. Keeping the blood spots dry with desiccant contributes to the stability of analytes.

Several community-based applications have shown this to be a convenient and reliable means to facilitate sample collection, storage, and transportation, and laboratory methods have been validated for a growing number of analytes [23,24,25,26,27]

Blood spotted on filter paper has been found to be suitable in large scale population screening programmes · The method has been used extensively for mass screening and for measurement of glucose[28], insulin, glycosylated hemoglobin [29] and steroids[30]

A study has been done to review the advantages of implementing the DBS technique in the HIV field, especially in resource-constrained regions.[31]

A study demonstrates the convenience and simplicity of using DBSs to assess Postprandial Insulin and Triglycerides after Different Breakfast Meal Challenges, which are the markers of postprandial dysmetabolism in an ambulant group of subjects after consuming a variety of breakfast meals[32]

Starck and Lovegren used 4°C stored blood specimens for 5-14 years for the analysis of sterols. They did not look at sterol levels in fresh samples and therefore could not evaluate the degree of degradation with storage.[33]

Kapur and colleagues reported the usage of dried blood for cardiometabolic risk factors, including triglyceride screening in patients at high risk.[34] The dried blood collection in this study was also under controlled laboratory conditions.

Quraishi *et al.*– have reported a triglyceride determination method in dried blood spots and have demonstrated that levels were stable for 30 days at 16–28°C and for 90 days at 4°C .[35]

Dried serum/blood on filter paper has been found suitable for measurement of insulin [36,37,38] C Reactive protein[39,40,41] Inflammatory markers, thyroid hormones[42], alpha fetoprotein, growth hormone, HIV-1 etc .

Scientific Director of ZRT Laboratory, Dr. Kapur has been instrumental in developing a technology called dried blood spot testing that is being utilized for early detection of major indicators associated with heart health. They have concluded that the procedure requires small volumes of sample and supports a reliable and safe specimen collection and delivery system at a significantly low cost. Moreover blood could also be obtained by capillary puncture, which is less invasive than venipuncture. The only caveats are that, analytes to be measured must be stable to drying and must be released from the paper upon elution. [43]

Numerous surveillance studies were undertaken to assess the risk factors of cardiovascular disease in an Indian industrial population.[44,45,46]

A surveillance study was done on the use of dried blood for measurement of cholesterol and triglycerides, in which dried blood was collected in field and transported to laboratory located in close proximity for analysis.[47]

Cholesterol and triglyceride measurements from dried blood have been reported previously under controlled laboratory conditions.[48,49,50]

A community-based cross-sectional study for NCD risk factor surveillance was conducted in six centers spread across the country viz. Ballabgarh, Chennai, Dibrugarh, Nagpur, Trivandrum, and New Delhi (IHBAS) from 2004 to 2005. They concluded that dried blood would offer an excellent method for collection of blood for measurement of cholesterol and triglycerides for population surveys.[51]

Blood spotted on filter paper has been found to be suitable in large scale population screening programmes [52,53,54]

A Study done by Mc Dade et al concluded that the ease of DBS collection alleviates constraints associated with sampling in clinical settings, increases the frequency with which samples can be taken, and expands the methodologic options for population-level health research.[8]

Dried blood spots (DBS)—drops of whole blood collected on filter paper from a finger Prick have been incorporated into a number of major data collection efforts in the United States as well as internationally, [55]

- Great Smoky mountains Study for analysis of Biomarkers like Androstenidione, CRP, DHEA-S, Corisol, EBV antibodies , estradiol, FSH, LH, testosterone, CRP, and HbA1C[55]
- Health and retirement study for analysis of CRP, HbA1C , total Cholesterol and HDL
- Los Angeles Family and Neighborhood Survey for analysis of CRP, EBV antibodies, HbA1C, Total Cholesterol and HDL
- National Longitudinal Study of Adolescent Health for analysis of CRP, HbA1C , total Cholesterol and HDL, EBV antibodies

Dried blood spot sampling represents such a method, and a growing number of population-based studies, internationally and in the United States, are adding DBS to data-collection protocols. For many biomarkers, DBS sampling provides a viable alternative to using venipuncture, particularly as the long list of analytes that can be quantified in blood spot samples grows.[55]

There is a need in our country for considering this method as there is a large rural population and less availability of resources and technical capacity.Method should be standardised by a responsible authority and quality materials should be provided. Basic knowledge regarding spotting and storage of samples should be given to healthcare workers.

MATERIALS AND METHODS

SOURCE OF DATA

Blood Samples at K.L.E's Dr Prabhakar Kore Hospital and Medical Research Centre, Belgaum.

STUDY DESIGN- Cross-sectional study

SAMPLE CALCULATION- Considering the mean and standard deviation values from previous studies done, At 95% confidence limit and 5% Tolerance level, the sample size will be 83. Considering contamination and loosing results, 100 samples were analysed.

INCLUSION CRITERION - Blood samples for lipid profile

EXCLUSION CRITERIA

- Hemolysed samples
- Samples of Jaundice patients with high bilirubin

APPROVAL FROM THE AUTHORITIES:

Permission to conduct the study was obtained from all the concerned authorities viz.

1. Institutional ethics committee on human subjects research of Jawaharlal Nehru medical college, Belgaum.
2. Permission from Principal and Medical superintendent of KLE'S Dr.Prabhakar Kore Hospital and Medical Research Centre, Belgaum.

METHODOLOGY

Blood Samples available at the laboratory were taken and serum was separated. Hemolysed samples were excluded.

Analysis was done according to the details mentioned by Lakshmy Ramkrishnan et al. [48]

An aliquot of fresh serum sample was analyzed immediately for Cholesterol and Triglyceride levels with the commercially available kits in a semiautoanalyser . From the remaining serum, exact 10-[micro] L replicates of the serum samples were spotted onto 3M Whatman filter paper kept on a nonabsorbent surface (thermacol) and left at room temperature for 1 h for drying.

After drying, one aliquot was eluted and analyzed on the day of collection. The remaining filter discs were kept in a sealed plastic bag to protect them from dust and moisture and stored at room temperature for different time periods.

At 7, 14, 21, 28 and 35 days, entire dried serum spots corresponding to 10 [micro]L were cut out with scissors and transferred to 1.0 ml of enzymatic reagent.

For estimation of Cholesterol in fresh serum, 20 [micro] L serum was added to 1 ml of the reagent and the reaction was carried out at 37^{o} C for 10 min.

For estimation of Cholesterol in dried serum on filter paper, 2 spots of 10 [micro] L were added to 1 ml reagent and the reaction was carried out for 30 min, according to Lakshmi Ramkrishnan et al[48] 10 min will not be sufficient for the reaction to reach completion. Analysis was done using a Erba semiautoanalyser.

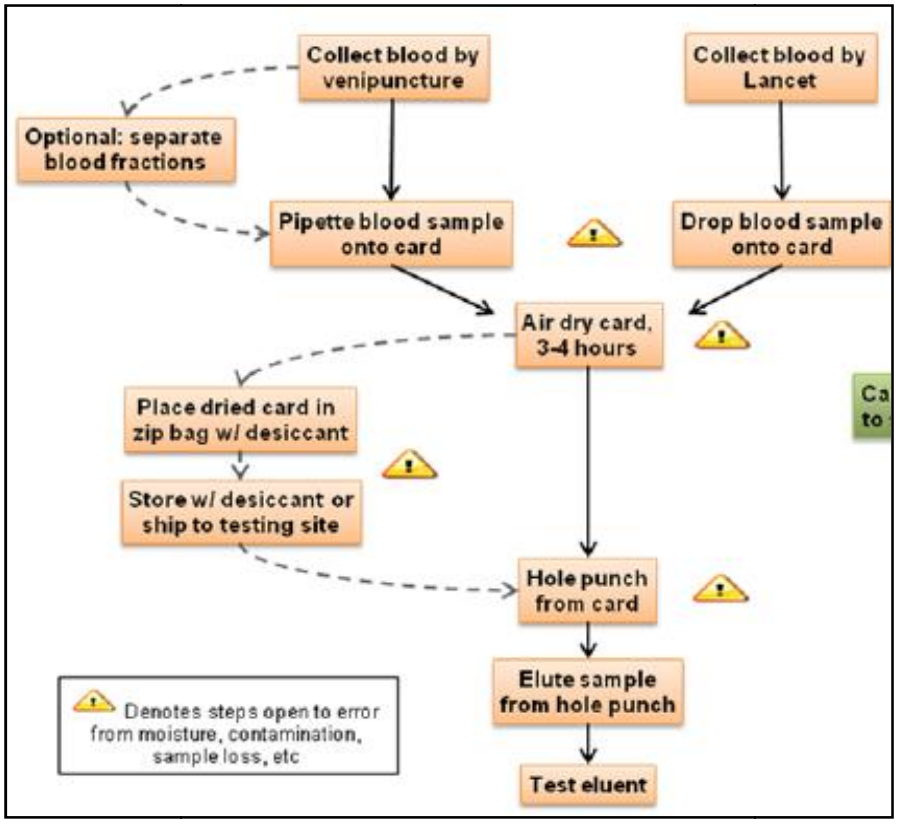
For estimation of triglycerides in fresh serum ,10 [micro] L was added to 1 ml of reagent and the reaction was carried out at 37^{o} C for 10 mins.

For estimation of Triglycerides in dried serum on filter paper, 1 spot of 10 [micro] L was added to 1 ml reagent and the reaction was carried out for 30 min. according to Lakshmi Ramkrishnan et al 10 min will not be sufficient for the reaction to reach completion. Analysis was done using a Erba semiautoanalyser.

Methanol can be used for elution of Cholesterol and Triglycerides from DBS.

METHOD FOR TRADITIONAL DRIED BLOOD / SERUM SPOT TECHNOLOGY

FIG 2



Precautions are needed for proper spotting, complete drying and proper storage to protect samples from dust, moisture and other contaminants. Careful cutting out of sample spots and complete elution of analyte has to be done.

ESTIMATION OF SERUM CHOLESTEROL:

Estimation of Serum Cholesterol was done by ERBA Semi Automatic analyzer using the commercially available ERBA Cholesterol kit, by Dynamic extended stability CHOD – PAP method, End point.

REAGENT COMPOSITION

REAGENT 1: CHOLESTEROL REAGENT

Cholesterol esterase (Pancreatic)	> 200 IU/L
Cholesterol oxidase (Microbial)	> 150 IU/L
Peroxidase	>2000 IU/L
Sodium Phenolate	20 mmol/L
4-aminoantipyrine	0.5 mmol/L
Phosphate buffer (pH 6.5)	68 mmol/L
Lipid clearing agent	

CHOLESTEROL STANDARD

Cholesterol Standard	200 mg/dL

AQUA 4

Double deionized 0.2 micron, membrane filtered, particle – free water for reconstitution of reagent 1

Total cholesterol measurement

The enzyme cholesterol esterase catalyzes the cholesterol ester to cholesterol and fatty acid. Addition of the enzyme cholesterol oxidase results in the formation cholest-4-en-3-one and H_2O_2. Addition of the enzyme peroxidase , phenol and 4-aminoantipyrine results in the formation of a colored compound (quiononeimine) that can be measured and its absorbance was determined at the wavelength of 505 nm. Absorbance of Quinonemine so formed is directly proportional to Cholesterol Concentration.

Cholesterol ester → Cholesterol + fatty acid (enzyme Cholesterol esterase)

Cholesterol + O_2 → cholest-4-en-3-one + H_2O_2 (enzyme Cholesterol oxidase)

2 H_2O_2 + 4AAP + Phenol → 4 H20 + Quinonemimine (Peroxidase)

Interfering substances for Cholesterol Estimation

Ascorbate: No significant interference up to 3 mg/dL Ascorbate

Bilirubin: No significant interference up to 8 mg/dL Bilirubin

Hemolysis: No significant interference up to 500 mg/dL Hemolysate

Lipemia: No significant interference up to 1000 mg/dL Intralipid

To avoid these interfering substances we excluded hemolysed samples, and the samples of patients with high bilirubin levels.

TABLE 3: ANALYSIS OF CHOLESTEROL LEVELS (AMERICAN HEART ASSOCIATION)[56]

Total Cholesterol Level	Classification
Less than 200 mg/DL	Desirable
200–239 mg/dL	Borderline-high risk
240 mg/dL and above	Very high risk

ESTIMATION OF SERUM TRIGLYCERIDES:

Estimation of Serum Triglycerides was done by ERBA Semi Automatic analyzer with the commercially available ERBA Triglycerides kit, by Dynamic extended stability with lipid clearing agent. GPO Trinder method, End point.

REAGENT COMPOSITION

ATP	2.5 mmol/L
Magnesium salt	2.5 mmol/L
4-aminoantipyrine	0.8 mmol/L
3-5 DHBS	1 mmol/L
Peroxidase	>2000 U/L
Glycerol kinase	>550U/L
GPO	>8000 U/L
Lipoprotein lipase	>3500 U/L
Buffer (pH 7.0 at 20^{o} C)	53 mmol/L

STANDARD TRIGLYCERIDE

Standard Triglyceride	200 mg/dL

AQUA – 4

TRIGLYCERIDE MEASUREMENT

The enzyme lipoprotein lipase catalyzes the triglycerides to glycerol and free fatty acids. Addition of the enzyme glycerokinase in presence of ATP results in the formation of glycerol-3-phosphate and ADP. Addition of the enzyme glycerol-3-phosphate oxidase results in the formation of dihydroxyacetone phosphate (DAP) and

H_2O_2. Addition of the enzyme peroxidase, 3,5,-Dichloro-2-hydroxybenzene sulfhonate (3,5 DHBS) and 4- aminoantipyrine results in the formation of a colored compound (quiononeimine) that can be measured and its absorbance was determined at the wavelength of 505 nm. Absorbance of Quinonemine so formed is directly proportional to Triglyceride Concentration.

Triglycerides + H_2O → Glycerol + free fatty acids

Glycerol + ATP → Glycerol-3-phosphate + ADP

Glycerol-3-phosphate + O_2 → DAP + H_2O_2.

H_2O_2 + 4AAP + 3,5 DHBS → Quiononeimine dye + 2 H_2O

Interfering substances for Triglyceride Estimation

Ascorbate: No significant interference up to 20 mg/dL Ascorbate

Bilirubin: No significant interference up to 40 mg/dL Bilirubin

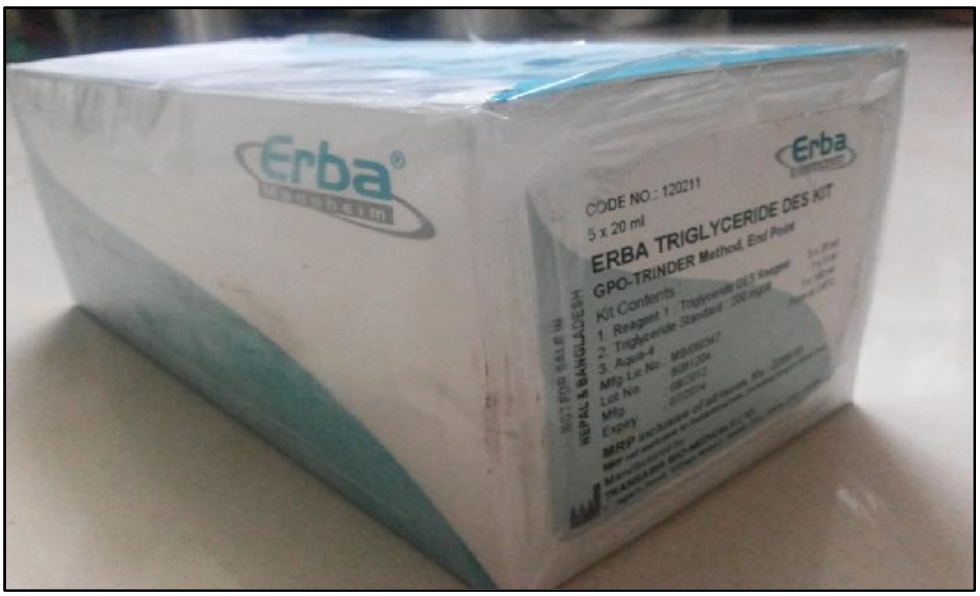
Hemolysis: No significant interference up to 500 mg/dL Hemolysate

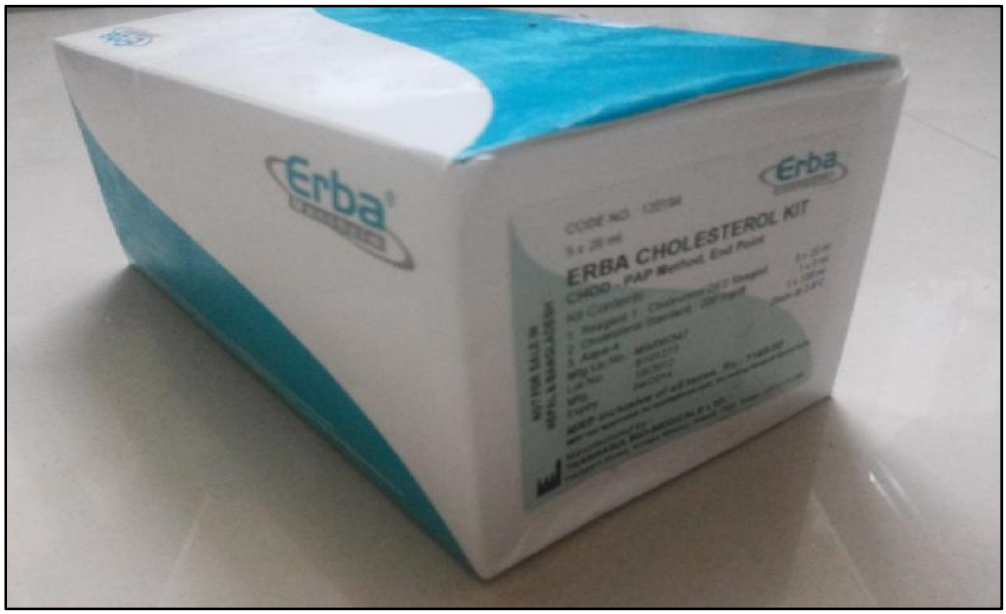
TABLE 4: ANALYSIS OF TRIGLYCERIDE LEVELS (AMERICAN HEART ASSOCIATION) [56]

Triglyceride Level	Classification
Less than 150 mg/dL	Desirable
150–199 mg/dL	Borderline-high risk
200–499 mg/dL	High risk
500 mg/dL or higher	Very high risk

Photograph 1: DRIED BLOOD SPOTS ON WHATMANN NO 3 FILTER PAPER

Photograph 2 & 3: CHOLESTEROL AND TRIGLYCERIDE KITS USED FOR ESTIMATION





Photograph 4: ERBA SEMIAUTOANALYSER

RESULTS

Cholesterol values in the 100 samples analyzed ranged from 102 mg/dl to 314 mg/dl.. The mean +/- standard deviation (SD) cholesterol values obtained from fresh serum was 148.33+/-30.68 mg/dl and the mean cholesterol values from corresponding dried serum was 147.86+/-30.67 mg/dl on the same day of drying and subsequently 147.59+/-30.47 (day 7) , 147.3+/-30.52 (day14) , 146.74+/-30.62 (day 21) , 146.45+/-30.69 (day 28) and 146.41+/-30.66 (day 35). **(Table 1)**

MEAN AND STANDARD DEVIATION FOR CHOLESTEROL (Table 5)

	Cholesterol (mg/dl)
Day 0(fresh)	**148.33+/-30.68**
Day 0(dried)	**147.86+/-30.67**
Day 7	**147.59+/-30.47**
Day 14	**147.3+/-30.52**
Day 21	**146.74+/-30.62**
Day 28	**146.45+/-30.69**
Day 35	**146.41+/-30.66**

Triglyceride values in the 100 samples ranged from 86 mg/dl to 168 mg/dl. The mean +/- standard deviation (SD) triglyceride values obtained from fresh serum was 113.18 + 18.77 mg/dl and the mean cholesterol values from corresponding dried serum was 112.78 + 18.62 mg/dl on the same day of drying and subsequently 112.52 + 18.63 (day 7) , 112.35 + 18.64 (day14) , 111.87 + 18.70 (day 21) , 111.63 + 18.72 (day 28) and 111.49 + 18.80 (day 35). **(Table 2)**

.

MEAN AND STANDARD DEVIATION FOR TRIGLYCERIDES (Table 6)

	Triglycerides (mg/dl)
Day 0 (fresh)	**113.18 + 18.77**
Day 0 (dried)	**112.78 + 18.62**
Day 7	**112.52 + 18.63**
Day 14	**112.35 + 18.64**
Day 21	**111.87 + 18.70**
Day 28	**111.63 + 18.72**
Day 35	**111.49 + 18.80**

Fig 1 shows the scatter plot of levels of cholesterol from all samples.

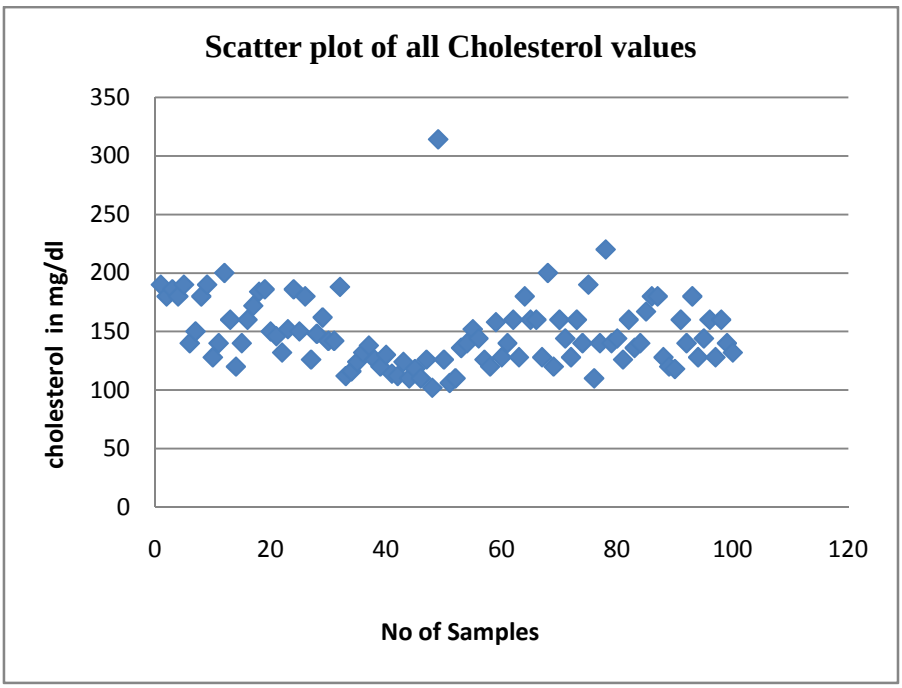
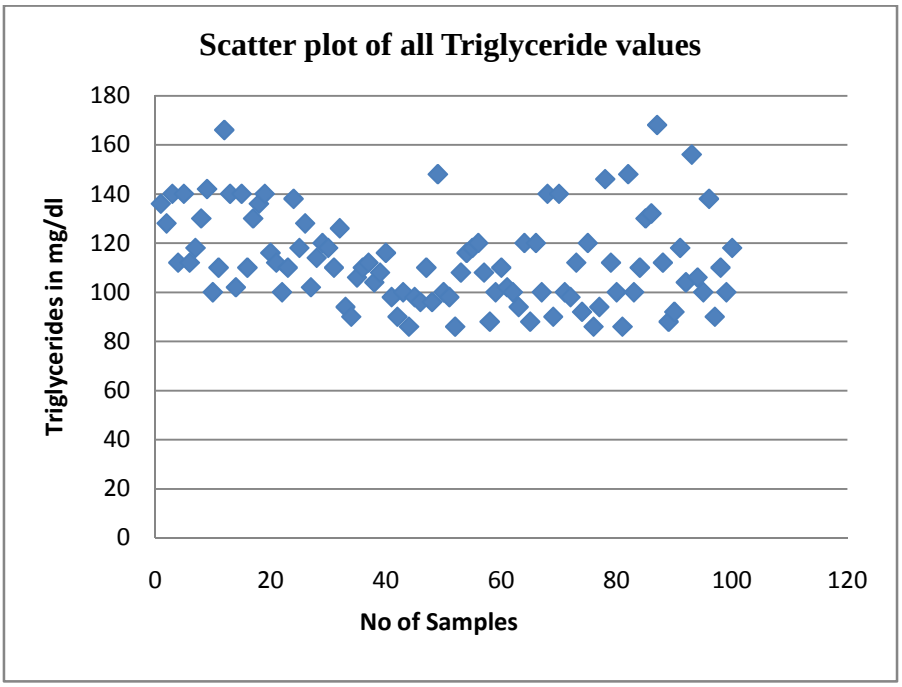


Fig 2 shows the scatter plot of all values of Triglycerides from all samples.



A Intra class correlation coefficient of 0.98 for cholesterol and 0.99 for triglycerides was evident between dried serum spots and fresh serum.

Bland Altman plots were done with Mean of Cholesterol vaues on x axis against differences in cholesterol values between fresh serum and dried serum on all (0,7,14,21,28 and 35)days

Bland–Altman plots suggest that the difference in values obtained by the two methods was within the 2 SD limits for most of the samples for Cholesterol (Figures 3,4,5,6,7 and 8) Less than 5% of the values were outside the 2 SD limits.[57]

Fig 3: Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 0

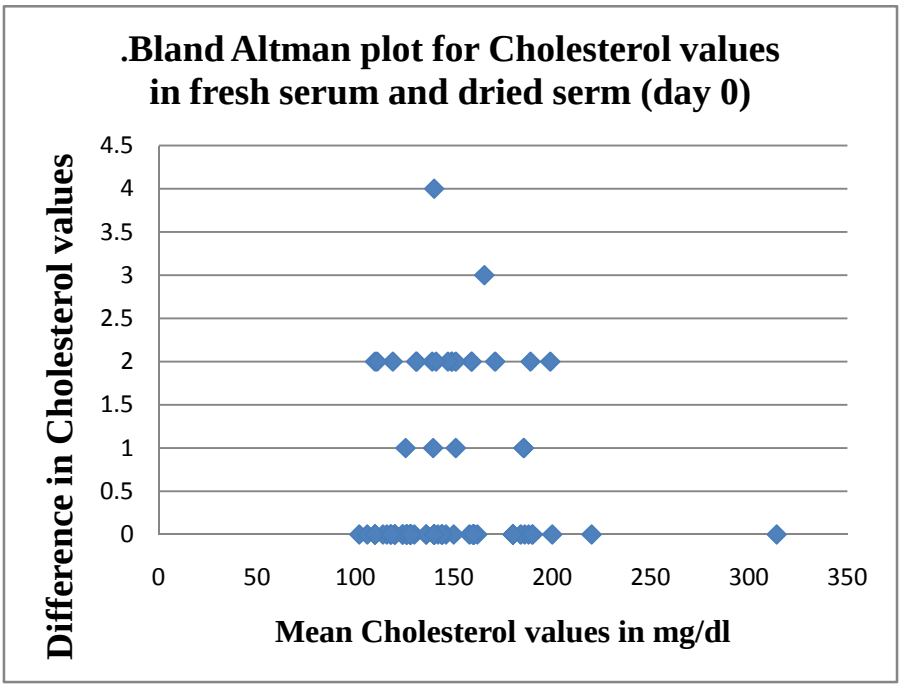


Fig 4 : Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 7

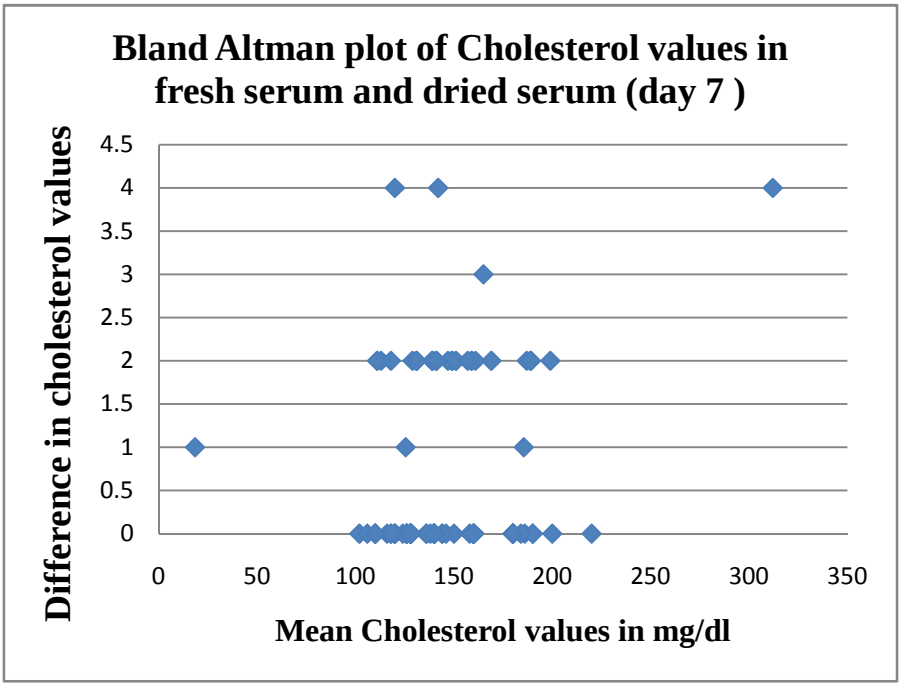


Fig 5 : Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 14

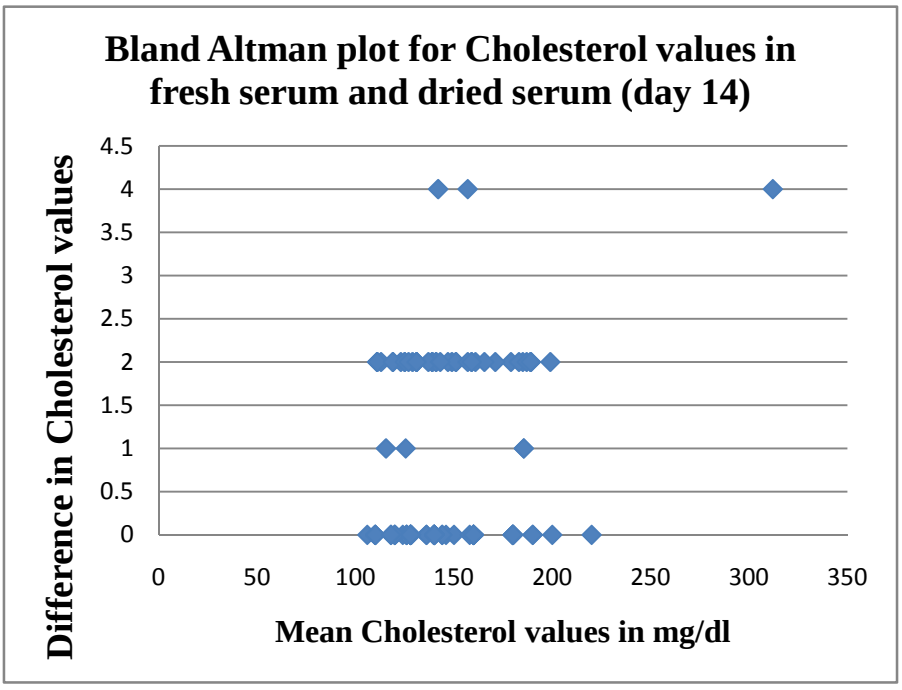


Fig 6 : Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 21

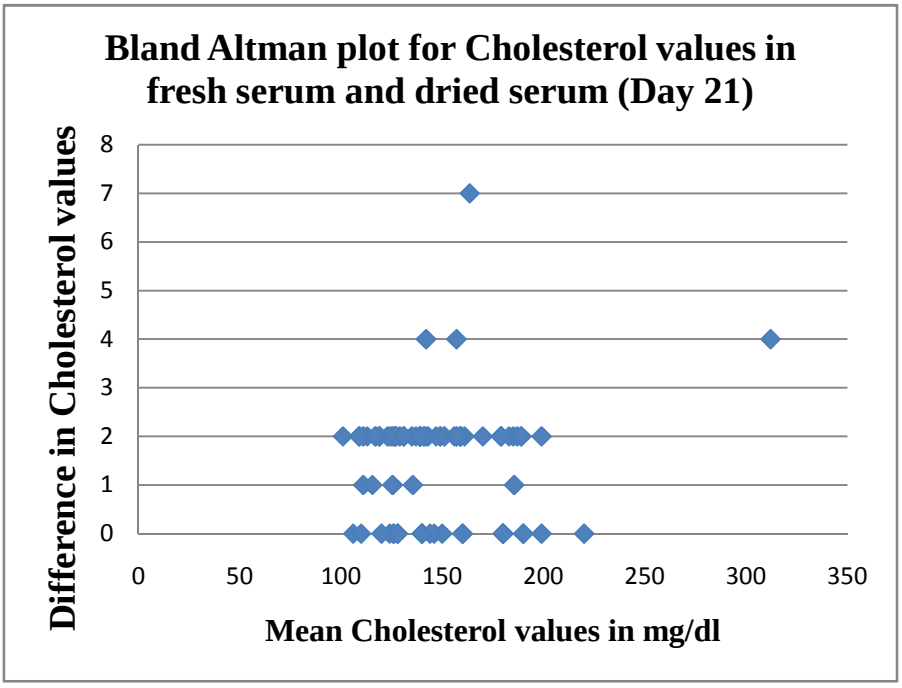


Fig 7: Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 28

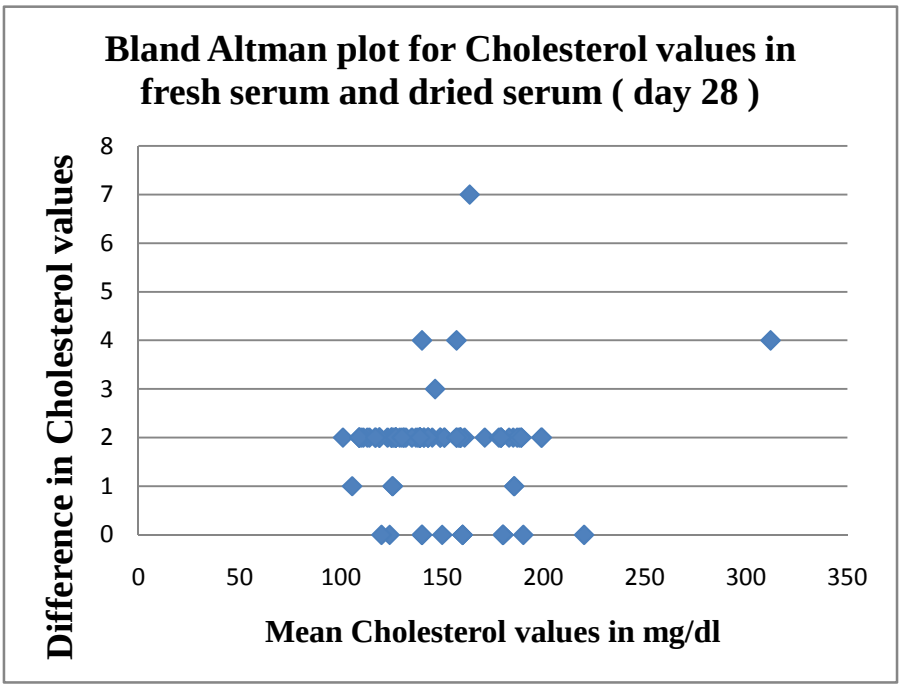
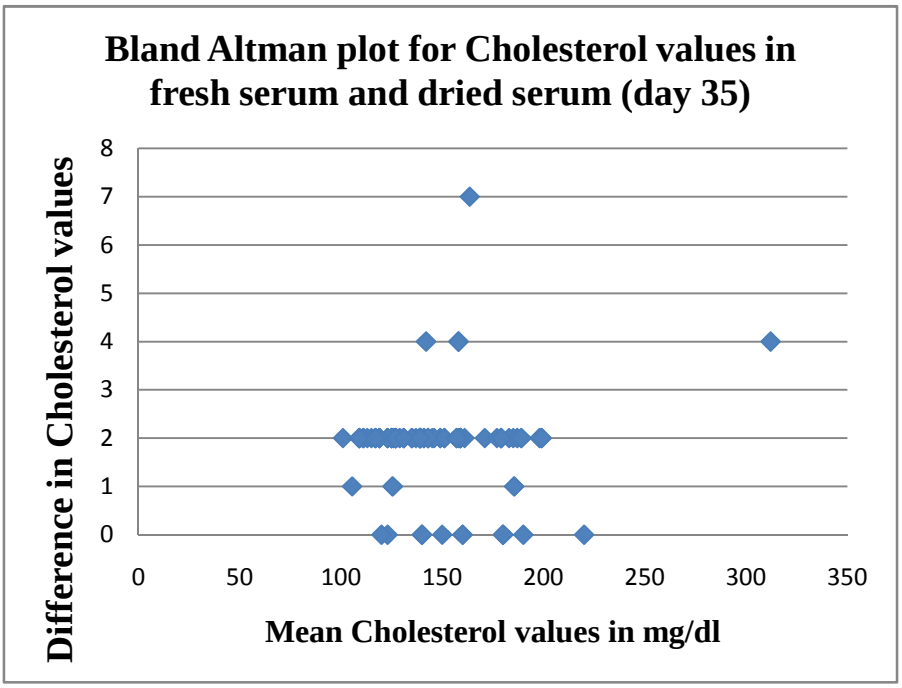


Fig 8 : Bland Altman plot of Mean Cholesterol values v/s differences in Cholesterol values between fresh serum and dried serum spot on day 35



Bland Altman plots were also done with Mean of Triglyceride vaues on x axis against differences in Triglyceride values between fresh serum and dried serum on all (0,7,14,21,28 and 35)days

Bland–Altman plots suggest that the difference in values obtained by the two methods was within the 2 SD limits for most of the samples for Triglycerides (Figures 9,10,11,12,13 and 14) Less than 5% of the values were outside the 2 SD limits

Fig 9: Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spot on day 0

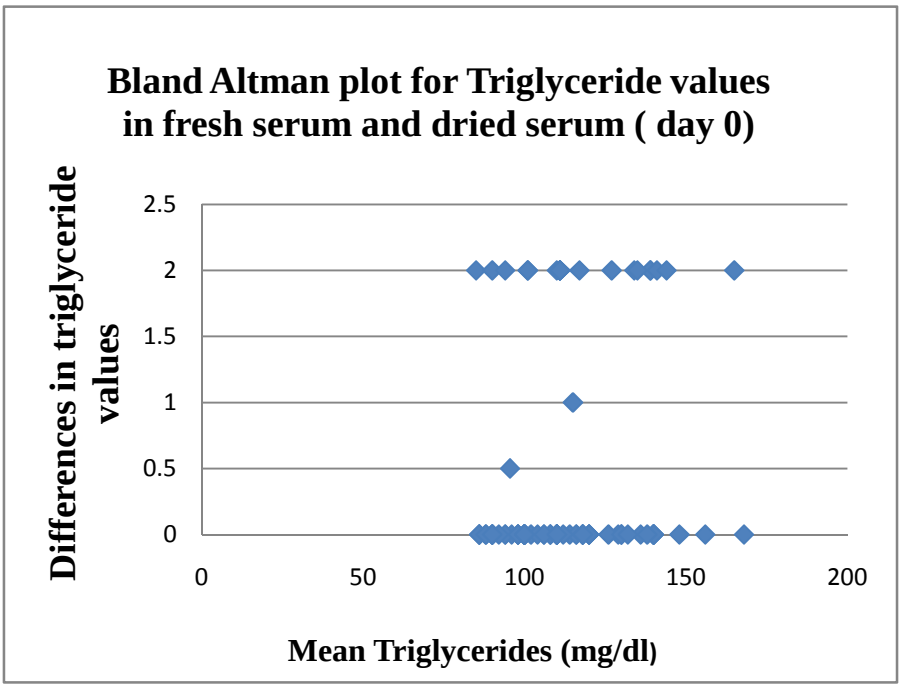


Fig 10 : Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spot on day 7

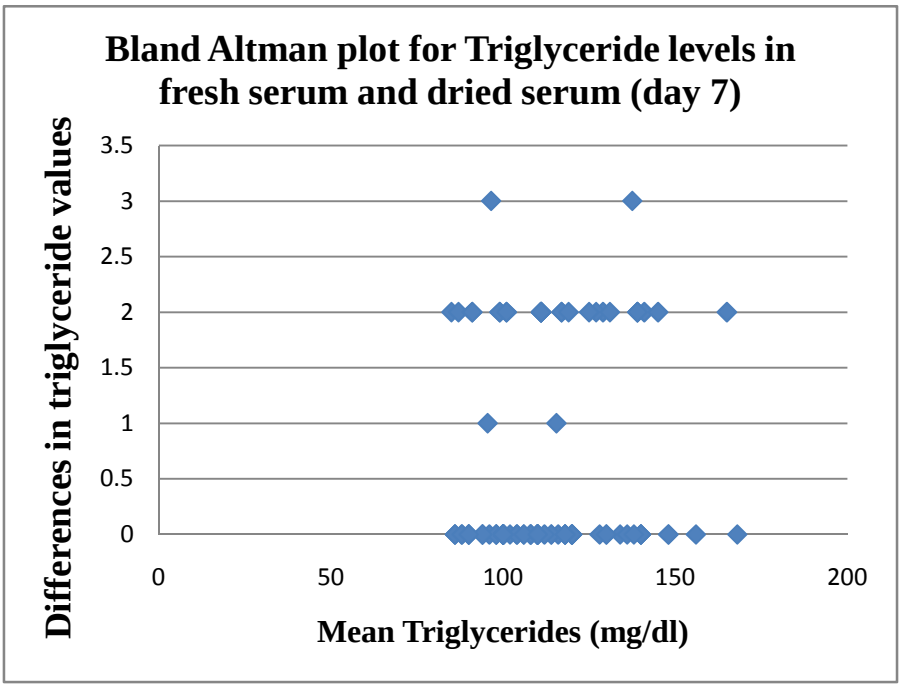


Fig 11: Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spots on day 28

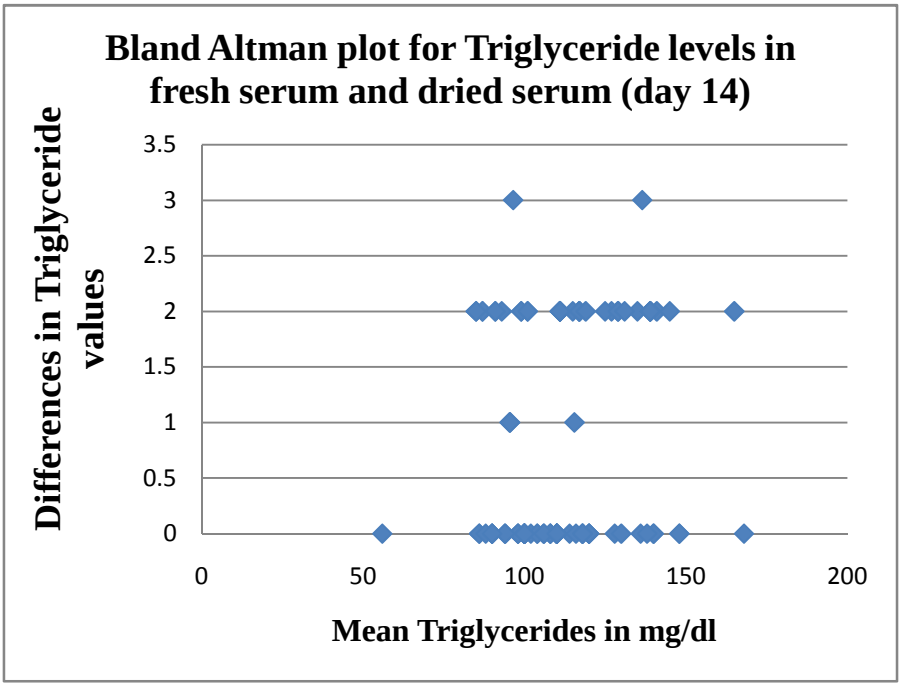


Fig 12 : Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spots on day 21

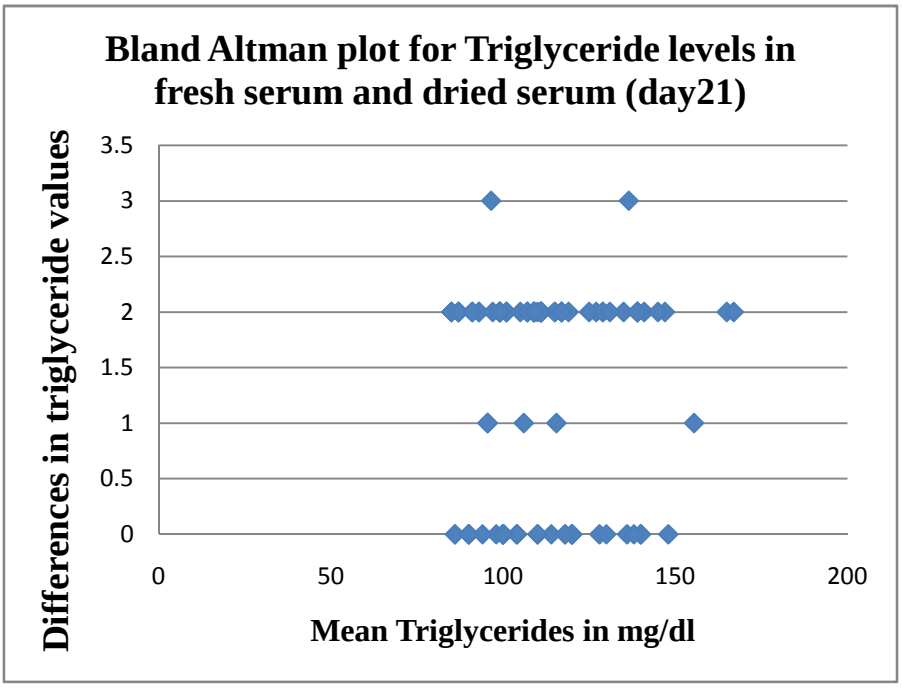


Fig 13 : Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spot on day 28

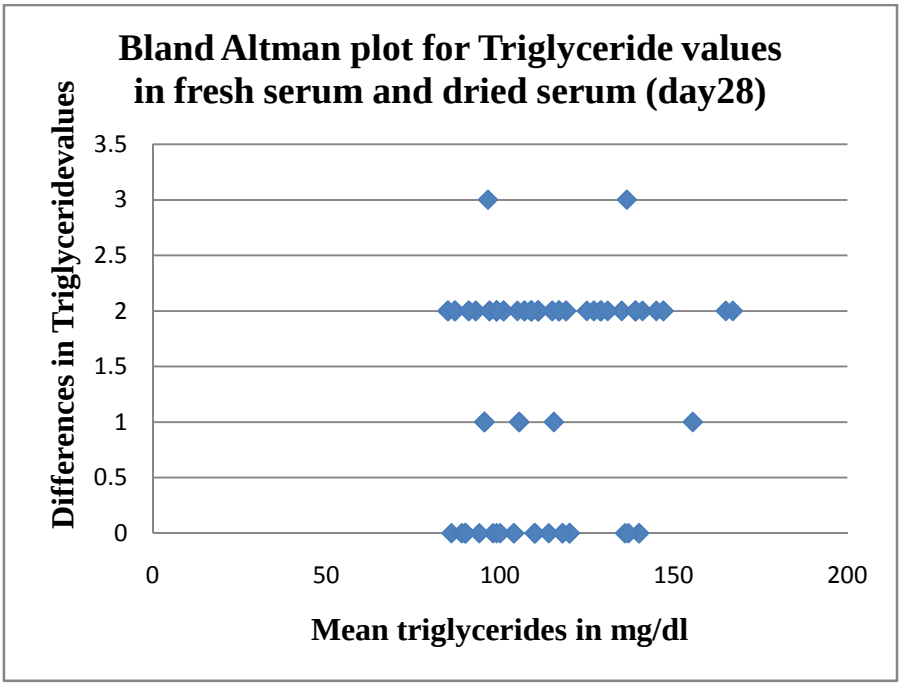
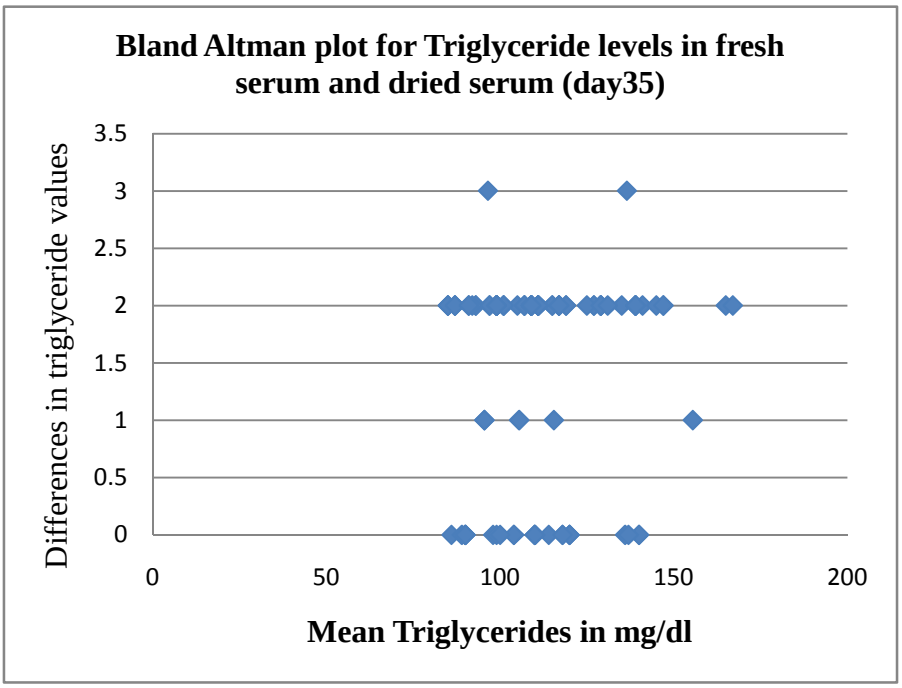


Fig 14 : Bland Altman plot of Mean Triglyceride values v/s differences in Triglyceride values between fresh serum and dried serum spot on day 35



Tonks and Barnett have published estimates of medically allowable error for critical decision levels. Tonks criteria are general (smaller of one fourth of the normal range or 10 % of the measured value . Barnetts recommendations are widely used but are limited to common laboratory analytes. Westguard has transformed Barnetts limits to 95% limits of allowable error i.e 95 % of patients should have errors less than the limit , or only 1 of every 20 samples can have an error larger than the specified limit. Accordingly medically allowable error for Cholesterol and Triglycerides is 40 according to Barnett and 25 according to Tonks.[58]

Our Study has given the values of Cholesterol and Triglycerides on Dried serum spots within medically allowable error, so it is acceptable.

DISCUSSION

Despite the clear benefits provided by DBS sampling, researchers are hesitant to adopt DBS methods for a number of reasons. The first is unpredictability: quite simply, fear of the unknown. The second reason is status quo bias: researchers are uncomfortable changing a method that is already working, even though greater efficiencies would result. The third reason is data comparability loss: concern for a potential discrepancy between historic plasma data vs. new DBS data. Finally, the lack of clear regulatory guidance: there is virtually no authoritative literature on the subject of changing from a plasma model to a DBS model.[59]

The analysis of dried blood/serum spot samples are similar to serum analysis, with some important changes. As the sample is dried on filter paper, analytes have to be brought into solution. The serum spots are cut out with scissors and put into an elution reagent for a fixed amount of time.

Whole blood comprises liquid and cellular fractions, and centrifugation of samples collected through venipuncture removes cellular components to yield serum or plasma. (Serum and plasma are comparable in this regard: the difference is that clotting factors have also been removed in serum.) When blood samples are dried on filter paper, cellular elements rupture, and their components are subsequently released into solution when DBS samples are reconstituted. Different assay systems and specific analytes will vary in their sensitivity to potential interference, and some assays may require additional processing prior to analysis. This is not a common problem, although the presence of lysed red blood cells has proven to be an insurmountable obstacle in the measurement of ferritin in dried whole blood[60]

Investigators can expect performance that is comparable to that obtained with serum/plasma samples, this may not always be possible. In such cases, the benefits of blood spot methods with regard to sample collection and handling will have to be weighed against the degree of potential error introduced during sample analysis. [55] Additional extraction procedures may be required for certain analytes to overcome this problem. Efficiency of elution of the analyte of interest and relative volume of sample collected are two other issues of concern in analysis with dried blood.

Minimization of preanalytical variations with proper collection of serum spots and storage are important determinants for the success of mass screening of total cholesterol and triglycerides using Dried serum/blood spots for risk factor assessment.

Furthermore, many standard clinical assays are performed on automated, high-throughput analyzers designed for use with serum or plasma samples. These instruments offer increased speed and reduced costs of analysis but currently are not likely to accommodate DBS samples.[55]

The use of dried blood/serum spots is ideal for pediatric applications and in multicenter studies where the costs and safety of sample transportation to a distant laboratory are limiting considerations. However, because serum from blood collected by venipuncture was used in the present study, this approach needs to be evaluated in whole blood from finger prick or heel prick.

Serum can be stored at -20°C in a non-self defrosting freezer for up to 4 weeks. For longer storage (> 4 weeks) they should be maintained at -80°C or lower. Total cholesterol and triglycerides are stable for at least one year at -80 ^{o}C or lower.

Whereas dried blood/serum spots can be stored at room temperature for upto 35 days, according to our study.

In a similar study conducted by Ramakrishnan Lakshmy et al at Delhi , they found that the correlation coefficient "*r*" was 0.78 for cholesterol and 0.94 for triglycerides between dried blood spots and serum. Bland–Altman plots suggest that differences in values obtained by the two methods were within two standard deviations for most of the samples.

In our study, we got comparable results, Cholesterol values in the 100 samples analyzed ranged from 102 mg/dl to 314 mg/dl.. The mean +/- standard deviation (SD) cholesterol values obtained from fresh serum was 148.33+/-30.68 mg/dl and the mean cholesterol values from corresponding dried serum was 147.86+/-30.67 mg/dl on the same day of drying and subsequently 147.59+/-30.47 (day 7) , 147.3+/-30.52 (day14) , 146.74+/-30.62 (day 21) , 146.45+/-30.69 (day 28) and 146.41+/-30.66 (day 35).

Triglyceride values in the 100 samples ranged from 86 mg/dl to 168 mg/dl. The mean +/- standard deviation (SD) triglyceride values obtained from fresh serum was 113.18 + 18.77 mg/dl and the mean cholesterol values from corresponding dried serum was 112.78 + 18.62 mg/dl on the same day of drying and subsequently 112.52 + 18.63 (day 7) , 112.35 + 18.64 (day14) , 111.87 + 18.70 (day 21) , 111.63 + 18.72 (day 28) and 111.49 + 18.80 (day 35).

A Intra class correlation coefficient of 0.98 for cholesterol and 0.99 for triglycerides was evident between dried serum spots and fresh serum.

Bland–Altman plots suggest that the difference in values obtained by the two methods was within the 2 SD limits for most of the samples for Cholesterol and for Triglycerides. Less than 5% of the values were outside the 2 SD limits.

We found that cholesterol and triglycerides are highly stable in dried serum and are readily transferable to a liquid phase. The good agreement between values in dried serum and fresh samples supports the validity of the measurement of these analytes in dried serum.

CONCLUSION

This study assessed the usage of dried serum spots for measurement of cholesterol and triglycerides, recognized as modifiable risk factors for cardiometabolic diseases. Dried blood spot collection has advantages compared to conventional blood draws, such as less invasive, less sample volume requirement, convenience of repeating measurements, and easy sample storage and transport. The comparable values between dried blood spot and serum assays supports the usage of dried blood spot sample collection method as an alternative when conventional venous blood draw facilities are not available or accessible.

The stability of cholesterol and triglycerides in dried blood samples at room temperature for 35 days ensures great applicability in developing countries with considerable rural populations who have limited accessibility to diagnostic labs performing the investigations. Further, the adaptation will be ideal for multicentric studies where the cost and safety of sample transportation to a distant laboratory are limiting factors.

The acceptable agreement between values in dried blood and fresh plasma samples supports the validity of the assay. However, precision and accuracy of the results can be improved by opting standard spotting method and proper storage.

The stability, efficient recovery, and excellent correlation with fresh serum samples makes the dried blood spot assay reliable and convenient method for screening modifiable risk factors like cholesterol and triglycerides.

Further scope of the study;

- A Study with dried blood spots from finger prick and heel prick can be done.
- A method is to be found, wherein analysis can be done on automated analysers.
- Collection of dried blood spots by finger prick, outside the laboratory conditions can be done.
- Storage temperature can be varied and tested
- Numerous other analytes can be studied.

SUMMARY

This study was undertaken to asses the possibility of using dried serum spots for Cholesterol and Triglyceride analysis.

100 samples in the laboratory of KLE, were analysed. An aliquot of each serum sample was analyzed immediately and exact 10-[micro] L replicates of the samples were spotted onto 3M Whattman filter paper kept on a nonabsorbent surface (thermacol) and left at room temperature for 1 h for drying. After drying, one aliquot was eluted and analyzed on the day of collection. The remaining filter discs were kept in a sealed plastic bag and analysed on 7, 14, 21, 28 and 35 days. For fresh serum, the reaction was carried out at room temperature for 10 min; for dried serum on filter paper, the reaction time was 30 min. Estimation of Cholesterol and Triglycerides was done with the commercially available kit in a semiautoanalyser.

A Intra class correlation coefficient of 0.98 for cholesterol and 0.99 for triglycerides was evident between dried serum spots and fresh serum.

Bland–Altman plots suggest that the difference in values obtained by the two methods (fresh and dried) were within the 2 SD limits for most of the samples for Cholesterol and Triglycerides. Less than 5% of the values were outside the 2 SD limits.

The comparable values between dried serum spots and serum assays supports the usage of dried blood spot sample collection method for estimation of Cholesterol and Triglycerides, as an alternative when conventional venous blood draw facilities are not available or accessible.

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MASTER CHART

Sample No	Cholesterol Day 0 (fresh)	Day 0 (dried)	Day 7	Day 14	Day 21	Day 28	Day 35
1	190	190	190	190	190	188	188
2	180	180	180	180	180	180	180
3	186	186	186	184	184	184	184
4	180	180	180	178	178	178	178
5	190	188	188	188	188	188	188
6	140	139	138	138	138	138	138
7	150	148	148	148	148	148	148
8	180	180	180	180	180	180	180
9	190	190	190	190	190	190	190
10	128	128	128	128	126	126	126
11	140	138	138	138	138	138	138
12	200	198	198	198	198	198	198
13	160	158	158	158	158	158	158
14	120	118	118	115	115	115	115
15	140	140	138	138	138	138	138
16	160	158	158	155	155	154	154
17	172	170	170	170	170	170	170
18	184	184	184	182	182	182	182
19	186	185	185	185	185	185	185
20	150	148	148	148	148	148	148
21	146	146	146	146	146	144	144

22	132	130	130	130	130	130	130
23	152	150	150	150	150	150	150
24	186	185	185	185	185	185	185
25	150	150	150	150	150	150	150
26	180	180	180	180	180	180	180
27	126	125	125	125	125	125	125
28	148	146	146	146	146	145	145
29	162	162	160	160	160	160	160
30	142	140	140	140	140	140	140
31	142	142	140	140	140	140	140
32	188	188	186	186	186	186	186
33	112	110	110	110	110	110	110
34	116	116	116	115	115	114	114
35	124	124	120	120	120	120	120
36	132	130	130	130	130	130	130
37	138	138	138	136	136	136	136
38	126	126	126	124	124	124	124
39	120	120	120	120	118	118	118
40	130	130	128	128	128	128	128
41	114	114	112	112	112	112	112
42	112	110	110	110	110	110	110
43	124	124	124	124	124	124	124
44	110	110	110	110	108	108	108
45	118	118	118	118	116	116	116

46	110	110	110	110	108	108	108
47	126	126	126	126	125	125	125
48	102	102	102	100	100	100	100
49	314	314	310	310	310	310	310
50	126	126	126	126	126	124	124
51	106	106	106	106	106	105	105
52	110	110	110	110	108	108	108
53	136	136	136	136	135	134	134
54	140	140	140	140	140	138	138
55	152	150	150	150	150	150	150
56	144	144	144	144	144	142	142
57	126	126	126	126	126	124	124
58	120	120	120	118	118	118	118
59	158	158	158	158	156	156	156
60	128	128	128	128	128	126	126
61	140	140	140	140	138	138	138
62	160	160	160	160	160	160	160
63	128	128	128	128	128	126	126
64	180	180	180	180	180	178	178
65	160	160	160	160	160	160	158
66	160	160	158	156	156	156	156
67	128	128	128	128	128	126	126
68	200	200	200	200	198	198	198
69	120	120	120	120	120	120	120

70	160	160	160	160	158	158	158
71	144	144	144	144	142	142	142
72	128	128	128	128	126	126	126
73	160	160	160	160	158	158	158
74	140	140	140	140	140	138	138
75	190	190	188	188	188	188	188
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77	140	140	140	140	140	140	140
78	220	220	220	220	220	220	220
79	140	140	140	140	138	138	138
80	144	144	142	142	142	142	142
81	126	126	126	124	124	124	124
82	160	160	160	160	158	158	158
83	136	136	136	136	134	134	134
84	140	140	140	140	138	138	138
85	167	164	164	164	160	160	160
86	180	180	180	180	178	176	176
87	180	180	180	180	180	180	178
88	128	128	128	128	126	126	126
89	120	120	120	120	118	118	118
90	118	118	118	118	116	116	116
91	160	160	160	158	158	158	158
92	140	140	140	140	140	140	140
93	180	180	180	180	178	178	178

94	128	128	128	128	126	126	126
95	144	140	140	140	140	140	140
96	160	160	160	160	160	160	160
97	128	128	128	126	126	126	126
98	160	160	160	160	156	158	158
99	140	140	140	140	140	140	140
100	132	130	130	130	130	130	130

Sample No	Triglyceride Day 0 (fresh)	Day 0 (dried)	Day 7	Day 14	Day 21	Day 28	Day 35
1	136	134	134	134	134	134	134
2	128	128	128	128	128	126	126
3	140	138	138	138	138	138	138
4	112	110	110	110	110	110	110
5	140	138	138	138	138	138	138
6	112	110	110	110	110	110	100
7	118	118	116	116	116	116	116
8	130	130	128	128	128	128	128
9	142	140	140	140	140	140	140
10	100	100	98	98	98	98	98
11	110	110	110	110	110	110	110
12	166	164	164	164	164	164	164
13	140	140	140	140	140	140	140
14	102	100	100	100	100	100	100
15	140	140	140	140	140	140	140
16	110	110	110	110	108	108	108
17	130	130	130	130	130	128	128
18	136	136	136	136	136	136	136
19	140	140	138	138	138	138	138
20	116	115	115	115	115	115	115
21	112	110	110	110	110	110	110

22	100	100	98	98	98	98	98
23	110	110	110	110	108	108	108
24	138	136	135	135	135	135	135
25	118	118	118	118	118	118	118
26	128	126	126	126	126	126	126
27	102	100	100	100	100	100	100
28	114	114	114	114	114	114	114
29	120	120	120	120	120	120	120
30	118	116	116	116	116	116	116
31	110	110	110	110	110	110	110
32	126	126	124	124	124	124	124
33	94	94	94	92	92	92	92
34	90	90	90	90	90	90	90
35	106	106	106	106	104	104	104
36	110	110	110	110	110	110	110
37	112	110	110	110	110	110	110
38	104	104	104	104	104	104	104
39	108	108	108	108	106	106	106
40	116	116	116	116	114	114	114
41	98	98	98	98	98	98	98
42	90	90	90	90	90	90	90
43	100	100	100	100	98	98	98
44	86	86	84	84	84	84	84
45	98	98	95	95	95	95	95

46	96	95	95	95	95	95	95
47	110	110	110	110	108	108	108
48	96	96	96	95	95	95	95
49	148	148	148	148	146	146	146
50	100	100	100	100	100	98	98
51	98	98	98	98	96	96	96
52	86	86	86	86	86	86	86
53	108	108	108	108	106	106	106
54	116	116	114	114	114	114	114
55	118	118	118	116	116	116	116
56	120	120	120	120	120	118	118
57	108	108	108	108	106	106	106
58	88	88	88	86	86	86	86
59	100	100	100	100	100	98	98
60	110	110	110	110	108	108	108
61	102	102	102	102	100	100	100
62	100	100	100	100	98	98	98
63	94	94	94	94	92	92	92
64	120	120	120	120	120	120	120
65	88	86	86	86	86	86	86
66	120	120	120	120	120	120	120
67	100	100	100	100	100	98	98
68	140	140	140	138	138	138	138
69	90	90	90	90	90	88	88

70	140	140	140	138	138	138	138
71	100	100	100	100	98	98	98
72	98	98	98	98	96	96	96
73	112	110	110	110	110	110	110
74	92	90	90	90	90	90	90
75	120	120	118	118	118	118	118
76	86	86	86	86	86	84	84
77	94	94	94	94	94	94	90
78	146	144	144	144	144	144	144
79	112	112	112	110	110	110	110
80	100	100	100	100	100	98	98
81	86	86	86	84	84	84	84
82	148	148	148	148	148	146	146
83	100	100	100	100	100	98	98
84	110	110	110	110	108	108	108
85	130	130	130	128	128	128	128
86	132	132	130	130	130	130	130
87	168	168	168	168	166	166	166
88	112	110	110	110	110	110	110
89	88	88	88	88	86	86	86
90	92	92	90	90	90	90	90
91	118	118	118	118	116	116	116
92	104	104	104	104	104	104	104
93	156	156	156	156	155	155	155

94	106	106	106	106	105	105	105
95	100	100	100	100	100	100	100
96	138	138	138	138	138	136	136
97	90	90	90	90	90	90	90
98	110	110	110	110	108	108	108
99	100	100	100	100	98	98	98
100	118	118	118	118	118	118	118

Introduction

Aims & Objective

Review of Literature

Materials & Methods

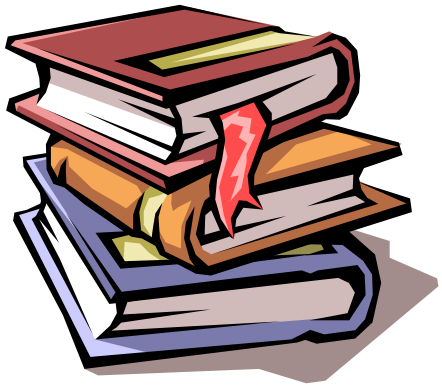
Results

Discussion

Conclusion

Summary

References



Annexure