1981

An Automated Method For The Determination Of Serum Triacylglycerols.

Louis J. Dunka Jr
University of Alabama at Birmingham

DUNKA, LOUIS, J., JR.

AN AUTOMATED METHOD FOR THE DETERMINATION OF SERUM TRIACYLGLYCEROLS

The University of Alabama in Birmingham PH.D. 1981

AN AUTOMATED METHOD FOR THE DETERMINATION OF SERUM TRIACYLGLYCEROLS

by

LOUIS J. DUNKA, JR.

A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biochemistry in The Graduate School, University of Alabama in Birmingham

BIRMINGHAM, ALABAMA

1981

ACKNOWLEDGEMENTS

The author wishes to express sincere gratitude to Dr. Leo M. Hall, his research advisor, and the graduate committee: Dr. S. B. Barker, Dr. E. L. Bradley, Dr. G. L. Carlson, Dr. J. M. McKibbin, and Dr. A. L. Siegel. Particular thanks is due Dr. Hall, without whose guidance, patience and wisdom this research never would have been completed. Special appreciation is expressed to my wife, Geraldine, for the many sacrifices made during the course of this research. The technical assistance of C. A. Rutledge is gratefully acknowledged. The assistance of the Biostatistics Department in the design and evaluation of the factorial experiments is gratefully acknowledged.

The author was supported by a Graduate Fellowship from the University of Alabama in Birmingham and a Career Development Fellowship from American Hospital Supply Corporation.

TABLE OF CONTENTS

TABLE OF CONTENTS (Continued) PAGE

LIST OF TABLES

LIST OF TABLES (Continued)

LIST OF TABLES (Continued)

LIST OF FIGURES

LIST OF FIGURES (Continued)

LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
BSP	Sulfobromophthalein
DG	Diglyceride
FC-134	Perfluoro alkyl quaternary ammonium iodide, available from Minnesota Mining and Manufacturing
GK	Glycerol kinase
HABA dye	2-(4'-hydroxyphenylazo)-benzoic acid
INT	Iodophenylnitrophenyltetrazolium chloride
LDH	Lactate dehydrogenase
mA	Milliabsorbance
MG	Monoglyceride
NAD^+	Nicotinamide adenine dinucleotide (oxidized form)
NADH	Nicotinamide adenine dinucleotide (reduced form)
PK	Pyruvate kinase
r	Pearson correlation coefficient
SD_D	Standard deviation of the difference
Sy	Standard deviation of the mean
TG	Triglyceride

CHAPTER I

INTRODUCTION

A. Definition of the Problem

Assays of serum triacylglycerol levels, in conjunction with serum cholesterol levels, have been found useful in the diagnosis of familial hyperlipoproteinemias. Elevated levels of serum triacylglycerols have been identified as a risk factor related to atherosclerotic disease. This latter fact has led to a dramatic rise in the number of assays for serum triacylglycerols performed by clinical laboratories. To meet this demand, various methods have been adopted to continuous flow analyzers to allow rapid assay of a large number of specimens.

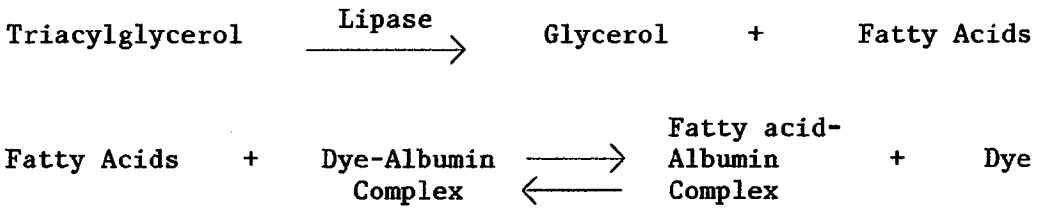
The early methods (Van Handel and Zilversmit, 1957; Kessler and Lederer, 1965) involved extraction of triacylglycerols into organic solvents. The extract was then subjected to alkaline hydrolysis. The glycerol liberated by hydrolysis was oxidized to formaldehyde and reacted to form various colored or fluorescent products. These methods were cumbersome and time consuming. More importantly, because of the extraction step, these methods were not amenable to total automation.

The use of enzymes, either after alkaline hydrolysis (Eggstein and Kreutz, 1966) or using enzymatic hydrolysis (Bucolo and David, 1973), greatly simplified the determination of serum triacylglycerols. However, because of the use of complex enzymatic coupling systems, the cost of these methods is high.

The goal of this research was the development of a direct, aqueous, spectrophotometric, automated method for the measurement of serum triacylglycerols. The proposed method was developed according to the following specific requirements which were considered necessary for its acceptance as a routine serum triacylglycerol assay method.

1. The proposed method must be specific for serum triacylglycerols.
2. The proposed method must be sensitive enough to use 200 µl or less of serum.
3. The proposed method must yield a response proportional to the concentration of serum triacylglycerols present.
4. The proposed method must be rapid enough to allow adaptation to continuous-flow instrumentation.
5. The reagents must be stable for several months on storage.
6. The method must be as inexpensive as possible.

The above requirements are met with a method that is based on the following reaction sequence:



The conditions chosen for the proposed method yield a direct relationship between serum concentrations of triacylglycerols and dye displaced from the dye albumin complex up to a serum triacylglycerol concentration of 500 mg/dl.

The proposed serum triacylglycerol reagent of this dissertation includes the following components: sodium pyrophosphate, bovine serum albumin (BSA), sulfobromophthalein (BSP), sodium azide, FC-134 surfactant and lipase from Rhizopus arrhizus.

B. Experimental Approach

The proposed serum triacylglycerol method was developed by optimizing a number of parameters that were found to have effects upon:

1. The magnitude of response, as measured by the absorbance change at 580 nm produced by a given amount of exogenous fatty acid.
2. The extent of linearity of response of the method to increasing levels of serum triacylglycerols.
3. The extent of hydrolysis produced by the lipase within the 4 minute incubation period in the heating bath.

4. The change in absorbance of the reagent due to binding of dye by albumin from serum specimens, hereafter referred to as the "protein effect."
5. The clearing of lipemic samples by lipase action, and the extent of error thereby introduced, hereafter referred to as the "turbidity effect."
6. The stability of the reagent.
7. The specificity of the proposed method.

Therefore, optimizing a particular parameter did not always involve assigning equal importance to each of the above considerations. In general, however, sacrifices in sensitivity were considered justified if significant improvements in (2) or (3) above were obtained.

The experimental parameters that were optimized for serum triacylglycerol measurement included the following:

1. A source of albumin was chosen for the dye-albumin complex by consideration of the extent of binding of dyes by the albumin, the reversibility of the binding, availability and cost. BSA was a clear-cut choice.
2. Using BSA, a variety of dye classes was screened. BSP was the dye which yielded the best combination of high molar absorptivity, strong binding affinity for BSA and reversibility of the binding when challenged by exogenous fatty acid.
3. Addition of small amounts of exogenous fatty acid to the reagent was found to be necessary if the plot of response

vs concentration of fatty acid in the sample were to pass through the origin.

4. The selection of BSP as the dye dictated that the reagent would have a pH of 7.8 or greater. Thus, the selection of a lipase was limited to those with a pH optimum in this range. This consideration, in conjunction with the needs for low cost and high specificity, led to the use of lipase from Rhizopus arrhizus. Lipase levels were varied to determine the optimum level necessary for all serum specimens to be analyzed, including lyophilized "standards."
5. FC-134 and sodium azide were chosen after evaluation of other surfactants and antimicrobial agents.
6. The pH and buffer salt were changed several times during the research to achieve different ends. The final pH of 8.0 is a compromise between two opposing considerations. As the pH is increased above 8.0, the sensitivity of color response to fatty acid increases rapidly; at the same time, as the pH is increased above 8.0, the amount of serum triacylglycerol hydrolysis per unit time by the lipase diminishes rapidly.
7. The "reaction" in the dye displacement step of the proposed method is actually competition between dye and fatty acid for binding sites on the albumin. For this reason, it is naive to assume that a simple variation of one component at constant levels of the other components

would yield an optimum concentration of that component. Thus, two factorial studies were performed. In the first study, pH, dye, albumin and "fatty acid for adjustment" levels were varied. In the second study, the pH was held constant at 8.0 and the other three parameters varied.

8. Throughout the later stages of the research, several variable parameters on the continuous flow instrument were systematically evaluated to establish the optimum manifold configuration. Once again, compromises were necessary, since maximization of one parameter resulted in deleterious effects on another.

9. The mode of "standardization" of the proposed method was evaluated by examination of three parameters:

   a. The serum triacylglycerol level and the level of endogenous glycerol in the "standard" were carefully determined.

   b. The effect of freezing and lyophilization on these values and on the values obtained by the proposed method were evaluated.

   c. The "turbidity effect" and the "protein effect" were determined on serum specimens which had been maintained in the liquid, frozen and lyophilized states to account for any errors introduced by the physical state of the specimen.

10. After selection of the best combination of concentrations and conditions as outlined above, a systematic evaluation

of the validity of the method was undertaken. Precision and accuracy were evaluated at various serum triacylglycerol levels. The magnitude of the protein and turbidity effects was determined, using samples chosen randomly. Finally, the agreement of the proposed method with two commercially available serum triglyceride methods was determined with serum specimens which represented a 30-fold range of concentration of serum triacylglycerols.

CHAPTER II

LITERATURE SURVEY

A. Rationale for Serum Triacylglycerol Measurements

"Alterations in serum lipoprotein concentrations may be secondary to a variety of endocrine, metabolic or other disorders, including thyroid, kidney and liver disease and diabetes mellitus, or they may be familial or genetically determined" according to Sanabar (1969). He further stated,

> Because of the difference in types and metabolism of serum lipoproteins, various clinical types or syndromes emerge, depending on which type of lipoprotein is principally abnormal. However there is substantial overlapping of symptoms and signs, because, with rare exceptions, atherosclerosis and its sequelae are the prevalent and most ominous complications in the majority of patients with hyperlipoproteinemia, leading to premature death from myocardial infarction or stroke.

Fredrickson and coworkers (1967), in a series of publications on fat transport by lipoproteins, provided a systematic set of criteria for the classification of metabolic disorders involving lipids and lipoproteins. The serum levels of cholesterol and triacylglycerols, the appearance of the serum after refrigerated, overnight storage and the lipoprotein patterns by electrophoresis

or ultracentrifugation are the basis for this system of classification. Havel (1969) has shown that the measurement of serum cholesterol and triacylglycerol levels along with appearance of the serum after refrigerated, overnight storage allows "lipoprotein diagnosis" of most patients. Albrink and Mann (1959), Albrink (1962) and Antonis and Bersohn (1961) have indicated preliminary evidence linking elevated levels of serum triacylglycerols to the development of atherosclerosis and heart disease. Deckelbaum, Tall and Small (1977) have shown, using X-ray scattering and calorimetry, that in very low density lipoproteins cholesterol esters are totally soluble in triacylglycerols, up to a molar ratio of 1:1. Smith (1975) has indicated the relationship between low density lipoprotein levels and the formation of atherosclerotic plaque. Thus, substantial reasons exist that show the need for accurate, precise and inexpensive methods for the determination of serum triacylglycerols. The fact that 2.5×10^7 serum triacylglycerol determinations were performed in the United States in 1976 (International Marketing Service Survey, 1977) shows the need for automation.

B. Triacylglycerol Methods

Early procedures for the determination of serum triacylglycerols were indirect, taking triacylglycerols as the difference between the total lipid content and the sum of the cholesterol esters, free cholesterol and phospholipid levels (Jacobs and Henry,

1962). The methods which have appeared in the literature since 1957 fall into one of five classes:

1. Non-enzymatic methods for the determination of glycerol after hydrolysis of the triacylglycerols.
2. Enzymatic methods for the detection of glycerol after hydrolysis.
3. Non-enzymatic methods for the detection of fatty acids after hydrolysis.
4. Totally enzymatic methods, employing glycerol detection.
5. Miscellaneous methods (infrared spectrophotometry, nephelometry and the hydroxamate reaction).

A tabulation of important methodologies in the historical development of serum triacylglycerol methods is presented in Tables I, II and III. The non-enzymatic glycerol detection methods may be broken down into four steps:

First, the lipids are extracted into an organic solvent.

Second, phospholipids, glucose and other serum components which can form glycerol or be oxidized to formaldehyde are removed, usually by adsorption.

Third, the phospholipid-free extract is either saponified or transesterified.

Fourth, either glycerol is determined after saponification, or ester groups by the hydroxamate reaction after transesterification. Several solvent-adsorbent systems have been used to extract triacylglycerols (and other lipids) from serum, and to remove possible interfering substances. A compilation of these combinations is

Table I

Survey of Methods for the Determination of Serum Triacylglycerols Using Chemical Methods

Author(s)	Extraction Solvent	Adsorbent	Indicator Reaction	Mode of Measurement	Degree of Automation	Moiety Measured
Van Handel and Zilversmit (1957)	Chloroform	Zeolite	Chromotropic tropic acid	Visible Spectrophotometer	Manual	Formaldehyde
Whitner, Mann and Witter (1972)	"	Silicic Acid	"	"	"	"
Carlson and Wädstrom (1959)	Chloroform Methanol	"	"	"	"	"
Vanzetti and Denegri (1964)	"	Florisil	"	"	"	"
Blankenhorn, Rauser and Weiner (1961)	Acetone-Ethanol	"	"	"	"	"
Azarnoff, Esker and Brock (1962)	Isopropyl Ether	Silicic Acid	"	"	"	"
Ryan and Rosho (1967)	Ethyl ether-Isopropanol	Florisil	"	"	"	"
Laurell (1966)	Isopropyl ether Ethanol	Silicic Acid	"	"	"	"
Rice (1970)	Chloroform-Methanol	"	"	"	"	"
Randrup (1960)	Hexane-Isopropanol	"	Phenylhydrazine-Ferricyanide	"	"	"
Jover (1963)	Chloroform-Methanol	"	"	"	"	"
Galletti (1967)	Isopropyl Ether	"	"	"	"	"
Dunsbach (1966)	Chloroform	Silicic Acid	Ammonium Acetate-Acetyl Acetone	Visible Spectrophotometer	Manual	Formaldehyde
Fletcher (1968)	Isopropanol	Zeolite + Lloyds Rgt + $Ca(OH)_2$ + $CuSo_4 \cdot 5H_2O$	"	"	"	"
Sardesia and Manning (1968)	Chloroform-Methanol	Silicic Acid	"	"	"	"

Table I - Continued

Author(s)	Extraction Solvent	Adsorbent	Indicator Reaction	Mode of Measurement	Degree of Automation	Moiety Measured
Malangeau and Pays (1967)	Isopropanol-Dichloro-methane	Zeolite	3-methyl benzothia zolin-2 one + $FeCl_3$	"	Manual	Formaldehyde
Mendelsohn and Antonis (1961)	Isopropyl Ether	Silicic Acid	o-aminophenol + arsenic acid + Mg^{++} + H_2SO_4	Fluorescence	"	"
Lofland (1964)	Isopropanol	Zeolite	Chromotropic acid	Visible Spec-trophotometer	Manual etha-KOH	"
Timms, et al (1968)	Isopropanol	See Fletcher	"	"	"	"
Antonis (1967)	Isopropyl ether + Acetic Acid	Silicic Acid	"	"	"	"
Yert, et al (1972)	Chloroform	Silicic Acid	"	"	"	"
Kessler and Lederer (1965)	Isopropanol	See Fletcher	See Dunsbach	Fluorometer	Isopropanolic KOH, Semi-automated	"
Cramp and Robertson (1968)	Isopropanol	Silicic acid + $CuSO_4 \cdot 5H_2O$ + $Ca(OH)_2$	"	"	"	"
Antonis (1967)	Isopropyl ether-acetic acid	Silicic Acid	"	"	"	"
Edwards, Falkowski and Chilcote (1972)	Isopropanol	See Fletcher	"	"	"	"
Royer and Ko (1969)	Nonane-isopropanol-0.08N H_2SO_4	----	"	"	Isopropanolic Sodium Methylate Semi-automated	"
Trout, Estes and Friedberg (1960)	Chloroform	----	----	Titration of fatty acids	----	Fatty acids*
Duncombe (1963)	"	----	Copper + dithiazone	Visible Spec-trophotometer	----	"*
Michaels (1962)	Chloroform-Methanol	Florisil	Hydroxamic Acid	"	----	Ester bonds
Freeman (1964)	"	Silicic Acid	----	Infrared trophotometer	----	"
Buckley, et al (1970)	----	----	----	Nephelometer	----	Intact Triacyl-glycerol molecule

* Fatty acid detection methods, not triacylglycerol detection methods

Table II

Survey of Methods for the Determination of Serum Triacylglycerols Using Partially Enzymatic Methods

Author(s)	Extraction Solvent	Adsorbent	Indicator Reaction	Mode of Measurement	Degree of Automation	Moiety Measured
Spinella and Mager (1966)	Chloroform-Methanol	Silicic Acid	Glycerol phosphate dehydrogenase	UV	Manual	Glycerol
Altmann, Bach and Metars (1967)	Chloroform	Zeolite	Glycerol + ATP + ADP + PEP Pyruvate + NADH	"	"	"
Schmidt and von Dahl (1968)	Chloroform-Methanol	----	"	"	"	"
Timms, et al (1968)	Isopropanol	Zeolite	"	"	"	"
Eggstein and Kreutz (1966)	Ethanolic KOH	----	"	"	"	"

Table III

Survey of Methods for the Determination of Serum Triacylglycerols Using Totally Enzymatic Methods

Author(s)	Method of Hydrolysis	Indicator Reaction	Mode of Measurement	Moiety Reacted	Degree of Automation
Bucolo and David (1973)	lipase + esterase	Glycerol + ATP + ADP + PEP Pyruvate + NADH	UV	Glycerol	Manual
Bucolo, Yabut and Chang (1975)	"	"	"	"	Totally automated
Klose, Munz and Wahlefeld (1977)	"	"	"	"	"
Stavrapolous and Crouch, (1974)	"	Glycerol Phosphate Dehydrogenase + INT + Diaphorase	Visible Spectrophotometer	"	Manual
Mazza and Crowther (1976)	Lipase	Glycerol Dehydrogenase + INT + Diaphorase	"	"	"

indicated in Table I. The solvent-adsorbent combination used by Fletcher (1968) has been widely used. The semi-automated method of Kessler and Lederer (1966), in which extraction and adsorption were performed manually, was widely used before the appearance of the enzymatic methods. The methods of Rice (1970) and Edwards, Falkowski and Chilcote (1972) were adopted as standard manual and automated methods respectively by the American Association of Clinical Chemists.

The first enzymatic glycerol-detection methods (Garland and Randle, 1962; Eggstein and Kreutz, 1966; Altmann, Bach and Metars, 1967) still involved a manual extraction and saponification, and use of an adsorbent in some cases. Bucolo and David (1973) achieved fully enzymatic measurement of serum triacylglycerols by use of a lipase from Rhizopus delemar and an esterase. The glycerol produced by this hydrolysis was quantitated using the reaction sequence:

$$\text{ATP} + \text{Glycerol} \xrightarrow[\text{Kinase}]{\text{Glycerol}} \alpha\text{-Glycerol Phosphate} + \text{ADP}$$

$$\text{ADP} + \text{phosphoenolpyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} \xrightarrow[\text{Dehydrogenase}]{\text{Lactate}} \text{Lactate} + \text{NAD}$$

Using the same components, the method was automated by Bucolo, Yabut and Chang (1975).

Spinella and Mager (1966) used the reaction sequence:

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerol kinase}} \alpha\text{-Glycerol Phosphate} + \text{ADP}$$

$$\alpha\text{-Glycerol Phosphate} + \text{NAD} \xrightarrow[\text{Dehydrogenase}]{\alpha\text{-Glycerol Phosphate}} \text{Dihydroxy Acetone Phosphate} + \text{NADH}$$

and Stavrapolous and Crouch (1974) used diaphorase and the dye 2-(p-iodophenyl)-3-p-nitrophenyl-5-phenyl-tetrazolium chloride coupled to the method of Spinella and Mager to allow measurement in the visible portion of the spectrum. Mazza and Crowther (1976) have used a tetrazolium dye and diaphorase to couple to the glycerol dehydrogenase reaction.

Trout (1960) and Duncombe (1963) have determined fatty acids by extraction into chloroform-methanol, followed either by titration or formation of a copper soap and determination of the organic soluble copper using dithizone. Although these two methods have not been used to measure serum triacylglycerols, they are included because they represent the current methods for determination of fatty acids, the entity which represents over 90% of the molecular weight of triacylglycerols.

Infrared spectrophotometry (Freeman, 1964) and nephelometry (Buckley et al., 1970) have been used to measure the intact triacylglycerol molecule. The hydroxamic acid reaction (Michaels, 1962) has been used to measure the ester linkages in triacylglycerols.

From the preceding discussion, it may be seen that a substantial body of work exists which is directed at the measurement of serum triacylglycerols. Yet, none of the methodologies cited totally fulfills the need for an accurate, precise, inexpensive, automated method. The non-enzymatic methods are not truly automated, since the extraction-adsorption step is performed manually. Additionally, the non-enzymatic methods suffer from a variety of methodological problems, as outlined by Whitner, Mann and Witter (1972).

The enzymatic methods, in particular the methods of Bucolo, Yabut and Chang (1975) and the method of Wahlfeld, Klose and Munz (1975), are amenable to automation. However, the cost of these methods is high, in the range of two dollars a test if a blank for glycerol is included. The other methods (infrared spectrophotometry, nephelometry and the hydroxamic acid reaction) are essentially curiosities as far as routine testing for triacylglycerols is concerned.

Since the expense of the enzymatic glycerol detection methods is primarily due to the costly enzymes and cofactors used in the coupling reactions, a more direct approach would be desirable. The preceding tabulation indicates that considerable attention has been devoted to the quantitation of the glycerol moiety of serum triacylglycerols, while little has been devoted to the quantitation of the fatty acid moiety. Thus, attention to the determination of fatty acids after hydrolysis of triacylglycerols by a lipase might provide a productive approach.

C. Review of the Binding Literature

Serum albumin binds many ligands, including some constituents of serum, such as bilirubin, bile salts, and fatty acids. Several exogenous ligands, such as metal ions, detergents, dyes and a variety of organic compounds, are bound (Steinhardt and Reynolds, 1969). Fatty acids are bound extremely tightly by albumin, with an association constant of 1.1×10^8 for oleic acid (Goodman, 1958).

Procedures for the determination of the number of basic groups on proteins by precipitation of dye by the protein were developed by several early workers (Chapman, Greenberg and Schmidt, 1927; Rawlins and Schmidt, 1930; Fraenkel-Conrat and Cooper, 1944). The approach of Scatchard and Black (1949) was applied to the binding of methyl orange, azosulfathiozole and amaranth (Klotz, 1946; Klotz and Walker, 1947; Klotz, Triwush and Walker, 1948), laying the groundwork for the determination of binding constants. This work also demonstrated the presence of sites on protein molecules with different affinities for the same ligand.

Cogin and Davis (1951) showed that fatty acids compete with methyl orange for binding sites on albumin. Klotz (1946) investigated the spectral shifts associated with the binding of bovine serum albumin to azosulfathiozole as well as Oranges I, II and III. In the same study, Klotz showed that the spectral shifts were not observed when sodium dodecyl sulfate, gelatin or bovine γ-globulin were added to solutions of the dyes. However, the addition of

competing ligands (carboxylic acids) partially reversed the spectral shift seen when albumin was added to the dyes.

Several dyes, including 2-(4'-hydroxyphenylazo)-benzoic acid (Rutstein, Ingenito and Reynolds, 1953) and bromocresol green (Beng and Kim, 1973) have been used to measure serum albumin concentrations. Sulfobromophthalein is bound strongly to serum albumin, with an association constant at one binding site of 1.7×10^7 (Baker and Bradley, 1965). Fatty acids compete with sulfobromophthalein for binding sites on albumin, while bilirubin and salts of bile acids do not (Kucerová et al., 1966). Hall (unpublished results) has found that the addition of lipase to serum in a solution of resazurin and bovine serum produced a spectral change proportional to the amount of serum added. Thus the potential for detection of fatty acids from serum triacylglycerols following hydrolysis by lipase has been demonstrated.

A fatty acid detection system would not intrinsically be able to discriminate among fatty acids from various sources. Since serum contains components other than triacylglycerols which contain a fatty acid moiety, the hydrolysis step must be specific for triacylglycerols. The use of extraction-adsorption to remove other fatty acid containing lipids is precluded, since the goal of this research is an automated serum triacylglycerol assay method. Thus the use of a specific lipase is necessary for the proposed method.

D. Review of Lipases

Lipases are available from various sources. A partial listing is given in Table IV. Lipases from most sources demonstrate maximum activity at slightly alkaline pH, although some lipases show maximal activity at acid pH. A few lipases have more than one pH optimum. The substrate specificity of lipases from various sources is diverse, in three respects:

1. With respect to the fatty acid composition of the triacylglycerol, both in terms of chain length and degree of unsaturation.
2. With respect to activity against triacylglycerols, as compared to diacylglycerols and monoacylglycerols.
3. With respect to other fatty acid containing compounds, such as phospholipids and cholesterol esters. A summary of the specificity of several lipases is shown in Table IV.

E. Commercially Available Automated Methods for the Determination of Serum Triacylglycerols

Two totally automated, enzymatic methods for the determination of serum triacylglycerols are commercially available. The method of Bucolo, Yabut and Chang (1975), commercially available from Calbiochem, uses lipase from Rhizopus delemar and an esterase to

Table IV

pH Optima and Substrate Specificities of Lipases From Various Sources

Lipase Source	pH Optimum	Chain Length Specificity	Acylglycerol Specificity	Other Specificity	Reference
Chromobacterium viscosum	3-10	C_6-C_{12} and Triolein	TG>DG>MG	Hydrolyzes Cholesterol Esters	Sugiura and Isobe (1974)
Rhizopus arrhizus	3.5 & 7.5	Long Chain C_{16} & C_{18}	TG>DG>>MG	----	Benzonana (1974)
Candida cylindraceae	5.2 & 7.2	C_{16} & C_{18}	TG>DG>MG	----	Benzonana and Esposito (1971)
Rice Bran	5.5 & 7.5	C_3	TG>DG>MG	----	Shastry and Raghavendra Rao (1971)
Aspergillus niger	5.6	C_{16} & C_{18}	TG>>DG>>MG	----	Fukumoto, Iwai and Tsujisaka (1963)
Rhizopus delemar	5.6	Short and long chain	TG>DG. (MG with esterase)	hydrolyzes prostglandin esters	Fukumoto, Iwai and Tsujisaka (1964)
Human Pancreatic	6-10 or 8.6	C_3-C_{18}	TG>DG>>MG	----	Mattson and Volpenheim (1968)
Aspergillus flavus	6.2	C_{12}	TG>>DG>>MG	----	Hoover, Laurentis and Gunetileke (1973)
Human Lipoprotein lipase	7.4-8.0	C_{16}-C_{18}	TG>DG>MG	----	Nillson-Ehle, Belfrage and Bergström (1971)
Corynobacterium acnes	7.5-9.0	C_3-C_{16}	TG>DG>MG	Does not hydrolyze phospholipids or cholesterol esters	Hassing (1971)
Staphylococcus aureus	8.0	C_{16} & C^{18}	TG>DG>MG	----	Vadehra (1974)
Geotrichium candidua	8.2	C_{16} & C_{18} cis-9 and cis-9, 12 unsaturated	TG>>DG>>MG	----	Jensen (1974)
Pseudomonas aeruginosa	8.9	C_3-C_{18}	TG>DG>MG	----	Finklestein, Strawich and Sonnino (1970)

achieve hydrolysis of serum triacylglycerols. The glycerol produced by this hydrolysis is dialyzed into a recipient stream containing the components of the indicator reactions previously described. However, endogenous ketoacids in the serum can cause baseline drift, by reaction with lactate dehydrogenase (Wahlfeld, Klose and Munz 1975). Wahlfeld, Klose and Munz (1975) have modified this system to remove baseline drift by addition of an NADH regeneration reaction sequence. This modification is commercially available from Boehringer Mannheim, using lipase from Rhizopus arrhizus and a plant esterase for hydrolysis. However, this modification is a single channel method, which does not permit correction for endogenous glycerol present in the sample.

CHAPTER III

SELECTION OF OPTIMUM ASSAY CONDITIONS

A. Materials

1. Chemicals:

Proteins - Serum albumins (Fraction V) from rat, horse, dog, cow, pig, chicken, goat, rabbit, ox and human were obtained from Sigma, as were lactalbumin, ovalbumin, β-globulin and γ-globulin. Bovine serum albumin (Fraction V) was also obtained from Nutritional Biochemical, and Reheis Chemical Companies.

Dyes - Evans blue, resazurin, congo red, cresol red, chlorophenol red, bromocresol green, bromophenol blue, indocyanine green, bromothymol blue, meta-cresol purple, fluorescein isothiocyanate, fluorescein, dichlorofluorescein, alizarin red S and naphthol yellow S were obtained from Eastman Organic Chemicals. Methyl orange, Orange I, crystal violet, naphthol blue black, Orange III, indigo carmine, azocarmine, acroflavin and brilliant cresyl blue were obtained from National Aniline and Chemical. Evans blue, Orange IV, buffalo black NBR, nile blue A, ponceau S, ponceau 3R and sudan III were obtained

from Allied Chemical. HABA dye was obtained from J. T. Baker. Coomassie brilliant blue was obtained from Schwarz Mann. Amaranth was obtained from City Chemical Corp. Malachite green was obtained from Sigma. Aniline blue black was obtained from Matheson, Coleman and Bell. Bromocresol purple was obtained from Fisher and BSP was obtained from Aldrich.

Fatty acids and derivatives - The following were obtained from Applied Science Laboratories: lauric, myristic, palmitic, myristoleic, linoleic, and linolenic, acids. Triolein, oleic, stearic and palmitic acids, and cholesteryl oleate were obtained from Sigma.

Lithium ricinoleate was prepared by hydrolysis of castor oil using lithium hydroxide, and recrystallization from water.

Miscellaneous chemicals - Sodium phosphate and sodium pyrophosphate were purchased from Malinckrodt. Sodium azide was obtained from Fisher. Sodium chloride was obtained from Sigma. Chloroform and methanol were obtained from Burdick and Jackson Laboratories. All other chemicals were of reagent grade or comparable quality, and were obtained from reputable suppliers.

Enzymes - Lipase from Rhizopus arrhizus was obtained as an ammonium sulfate suspension from Fermco Biochemics. Lipase from Candida cylindraceae was obtained from Worthington Biochemicals. Lipase from Rhizopus delamar was

obtained from Miles laboratories. Lipase from Chromobacterium viscosum was obtained from Toyo-Jozo Chemical Co. Lipase from Geotrichium candidum was obtained from Sigma, as was lipase from porcine pancreas. Phospholipase A from Bee Venom (> 1500 U/mg) was obtained from Sigma. Cholesterol esterase and oxidase were obtained from Beckman Microbics.

Surfactants - The sources of surfactants are listed in Table VII.

2. AutoAnalyzer supplies - Tubing, dialyzers, filters and other supplies for the AutoAnalyzer II^{R} were purchased from either Technicon or Alpkem.

3. Serum specimens - Human serum specimens were obtained from two local hospitals and a local clinical laboratory. Once obtained, the specimens were stored refrigerated or frozen until use.

B. Methods

1. General Methods

Preparation of Experimental Variations of the Proposed Reagent. The reagent was prepared by adding buffer to distilled water, then adjusting to within 0.2 pH of the final pH. BSA and sodium azide were added as powders. BSP was added as a 20 mg/ml aqueous solution, and oleic acid as a 0.02 M methanolic solution. Final adjustment of pH after dilution was within ± .01 of the desired pH value.

Triacylglycerol Assay Methods Used for Methods Comparison. Two commercially available serum triacylglycerol assay methods were used to measure the triacylglycerol content in serum specimens.

The first method, commercially available from Calbiochem-Behring (Enzymatic Triglycerides-Glycerol^{R}), is based on the reaction sequence:

$$\text{Triacylglycerols} \xrightarrow[\text{Esterase}]{\text{Lipase}} \text{Glycerol + Fatty Acids}$$

$$\text{Glycerol + ATP} \xrightarrow[\text{Kinase}]{\text{Glycerol}} \alpha\text{-Glycerol Phosphate + ADP}$$

$$\text{ADP + Phosphoenol Pyruvate} \xrightarrow[\text{Kinase}]{\text{Pyruvate}} \text{ATP + Pyruvate}$$

$$\text{Pyruvate + NADH} \xrightarrow[\text{Dehydrogenase}]{\text{Lactate}} \text{Lactate + NAD}$$

The decrease in the absorbance at 340 nm is proportional to the triacylglycerol content of the sample. Omission of the lipase allows measurement of endogenous glycerol by this method.

The second method, commercially available from Harleco (Tri-ES^{R}), is based on the reaction sequence:

$$\text{Triacylglycerols} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty Acids}$$

$$\text{Glycerol + NAD} \xrightarrow[\text{Diaphorase}]{\text{Glycerol Dehydrogenase}} \text{NADH + Dihydroxyacetone}$$

Iodiphenylnitrophenyltetrazolium Formaxan chloride

The increase in absorbance at 505 nm is proportional to the triacylglycerol content of the sample. Correction for endogenous glycerol is not possible by this method.

Both methods were used according to the manufacturer's instructions, except that both sample and reagent volumes were reduced by one-third for the Calbiochem method. A Gilford System Four Computer Assisted Analyzer was used to perform the assays. All other spectrophotometric assays were performed on either the Gilford System 4, or a Hitachi 100-60 Dual Beam spectrometer, using a 1.0 cm light path.

Determination of Lipase Activity. The activity of the lipase from Rhizopus arrhizus obtained from Fermco Biochemics was measured using the Calbiochem Enzymatic Lipase Kit according to the manufacturer's instructions. The activities obtained on three lots of lipase correlated well with performance in the proposed method.

Protein Defatting Procedures. Certain experiments early in the development of the proposed method required BSA from which endogenous fatty acids had been removed. Three methods

were used at various times: The isooctane-acetic acid procedure of Goodman (1957); the charcoal-acid method of Chen (1967); or the ion exchange method of Reynolds, Herbert and Steinhardt (1968).

2. Methods Used to Select Optimum Conditions

Albumin Selection. Reagent variations were prepared by dissolving 3.45 g/l of each albumin in a reagent composed of BSP, 0.2 g/l in 0.2 M pH 7.8 sodium phosphate. The absorbance at 580 nm of each solution was determined before and after the addition of 1.0 μmole of a methanolic solution of oleic acid to 3.0 mls of the reagent in a 1.0 cm spectrophetometer cell. Those albumin specimens which demonstrated a positive absorbance change of 0.10 or greater using the above protocol were considered acceptable.

Dye Selection. Appropriate concentrations of various dyes, ranging from 4.5×10^{-4} M to 8.3×10^{-6} M, were dissolved in 0.20 M sodium phosphate buffer, pH 7.8, containing 3.40 mg/ml of BSA. Difference absorption spectra were obtained for each dye-reagent mixture, using the dye-reagent mixture as blank for the same dye reagent mixture to which 1.0 μmole of methanolic oleic acid per 3.0 ml of dye reagent mixture had been added. An acceptable dye was defined as one which demonstrated an absorbance change of 0.03 or greater upon the addition of oleic acid to the dye-reagent mixture in a 1.0 cm spectrophotometer cell.

Preadjustment of Reagent Response to Fatty Acid. In order to obtain a linear response of the reagent to the addition of increments of exogenous fatty acid, small amounts of oleic acid were added to the reagent. The addition of the proper amount was evaluated by addition of several levels of fatty acid to the reagent, and evaluating the regression line for response upon addition of fatty acid. The level of fatty acid which yielded the intercept closest to zero was chosen as the proper level.

Evaluation of Response of the Albumin-dye Complex Reagent to the Addition of Various Naturally Occurring Fatty Acids. Using the reagent formulation described in the legend to Figure 5, the response of the reagent was evaluated as follows: To 3.00 ml of reagent at 37.5°C, differing amounts of various fatty acids were added. The increase in absorbance at 580 nm due to fatty acid addition was measured. Oleic, palmitic, stearic, myristic and lauric acids were added as methanolic solutions. Lithium Riciocleate acid was added as an aqueous solution.

Evaluation of Surfactants. To achieve proper flow characteristics on continuous flow analyzers, a surfactant is commonly added to the reagents. An evaluation of surfactants for compatibility with the proposed method was conducted as follows:

The surfactant was added to 3.00 ml of reagent at a concentration of either 10 g/l or 10 ml/l of reagent, depending

on the physical state of the surfactant. The absorbance at 580 nm was determined before and after surfactant addition. One μmol of oleic acid was then added and the absorbance determined again. As a control, water was added instead of a surfactant. A surfactant was considered acceptable for further evaluation if:

a. No increase or decrease in absorbance was observed upon surfactant addition beyond the effect due to dilution.

b. The absorbance change upon fatty acid addition was the same in the presence or absence of surfactant.

c. No turbidity or precipitation was observed upon addition of the surfactant to the reagent.

If a surfactant satisfied the above criteria, a second set of experiments was performed. To a cuvette containing 3.00 ml of reagent and the appropriate concentration of surfactant, lipase was added and an absorbance reading taken after 5 minutes. Fifty μl of serum were then added and another absorbance reading taken after a time sufficient for total hydrolysis of serum triacylglycerols with no surfactant present. Two further criteria were then applied to assess the acceptability of the surfactant:

d. Absorbance change due to lipase action on the surfactant was negligible.

e. The extent and rate of hydrolysis of serum triacylglycerols were the same in the presence and absence of the surfactant.

If all of the above criteria were satisfied, the surfactant was evaluated for its performance as a wetting agent by observation of the bubble pattern on the AutoAnalyzer II^{R}, with reagents flowing through the lines.

Selection of an Antimicrobial Agent. Evaluation of antimicrobial agents was performed by adding various amounts of the agent, ranging from 0.1% (w/v or v/v) to 0.5% (w/v or v/v). The reagent was then stored at room temperature and 37°C for several weeks to several months. Periodically, the reagent was inspected to determine the presence of color change, turbidity, or sign of microbial growth. If none of these conditions was present, the performance of the reagent was determined and compared to freshly prepared reagent.

Lipase Selection. The choice of a lipase was governed by three considerations. First, the lipase must have substantial activity in the pH range 7.5 to 8.5, due to the pKa of BSP. Second, the lipase must be free of either cholesterol esterase or phospholipase activities. Third, the lipase must be available at low cost. It was desirable, but not necessary, that the lipase catalyze complete hydrolysis of triacylglycerols. As long as the degree of hydrolysis was consistent, the lipase was considered useful.

Factorial Studies. Using the AutoAnalyzer II^{R}, two factorial studies were performed to determine optimum reagent concentrations and conditions. In the first study, pH, dye level, BSA level and fatty acid level for preadjustment were varied. Lithium ricinoleate was used as a sample at five levels, corresponding to 300 to 1500 mg/dl triacylglycerol in equal increments. All variations of reagent composition were run at a constant colorimeter gain of 75, so that relative responses could be compared.

Trend analysis was performed to choose the optimum levels of the components varied. Regression statistics were calculated for each of the 84 separate combinations of component levels. Those combinations yielding the greatest slopes for the regression lines were plotted, and the optimum combination was chosen from these plots. The single combination which yielded the greatest sensitivity by this graphical method also yielded a very small intercept on the y-axis, and was thus an unequivocal choice.

A second, more limited factorial study was performed, varying dye, albumin and fatty acid for preadjustment levels. In this study, the colorimeter gain was set as described under standardization below. Serum pools with values of 145 through 740 mg/dl triacylglycerol were used as samples. As in the previous study, once the colorimeter gain was set, it was left constant throughout the study. Triplicate determinations were performed on each sample of pooled sera at each reagent concentration.

Triolein Hydrolysis. Triolein hydrolysis experiments were conducted as follows: At pH 7.8, to 3.0 ml of a reagent containing 4.2 g/l BSA, 200 mg/l BSP, 2 g/l sodium azide, 14 μmol/ml lithium ricinoleate and 15 ml/l of FC-134 in 0.2 M sodium phosphate were placed in a cuvette. Fifty μl of 3 mg/ml isopropanolic solution of triolein were added. The solution was then immersed in an ultrasonic bath for 30 to 60 seconds to insure total dispersion of the triolein. The absorbance of the solution at 580 nm was determined before and after triolein addition. To the solution containing triolein, were added 1600 U/l of lipase from Rhizopus arrhizus. Absorbance readings were taken at 5, 10 and 15 minutes after lipase addition. Using data from fatty acid response curves, and the known stoichiometry of hydrolysis of triolein to 3 moles of oleic acid, the percent hydrolysis was determined.

Standardization. Standardization of the proposed method was accomplished using serum pools. The serum pools were assayed by the Calbiochem method and the triacylglycerol value corrected for endogenous glycerol, if present. The colorimeter gain was then used to adjust the recorder response on the AutoAnalyzer II^{R} to the assay value obtained by this procedure.

Evaluation of the effects of lyophilization and freezing on the usefulness of pooled sera as standards for the proposed method was performed. Two samples of pooled sera were separated into three aliquots each. One aliquot was stored

refrigerated in the liquid state, one was frozen and one was lyophilized. Each aliquot of each pool was assayed, in duplicate, by the Calbiochem method for triacylglycerol content and corrected for endogenous glycerol.

Twenty-four randomly selected serum specimens were assayed for triacylglycerol content by the Calbiochem method and corrected for endogenous glycerol. This group of specimens was then assayed by the proposed method as follows: Each of the treatments of each serum pool described previously was used to set the colorimeter gain to a value which yielded the corrected Calbiochem assay value for that treatment. Thus, a total of six runs was performed, standardizing once with each treatment of each pool. Regression statistics were calculated for each treatment versus the Calbiochem values.

3. Evaluation of the Proposed Method

The AutoAnalyzer II^{R} system, comprising a Sampler II, a peristaltic proportioning pump, two 37° incubators, and two dual channel colorimeters along with a strip chart record was assembled from standard Technicon parts and operated according to the manufacturer's instructions, the recommendations of Thiers and Oglesbly (1964) and those of Furman (1976).

Linearity and Recovery Studies. Linearity and recovery studies were performed as follows: Normal and elevated serum pools were prepared and assayed by the Calbiochem method as in Standardization. Various mixtures of the pooled sera were

assayed repetitively. The response obtained was compared with the expected response calculated from mixtures.

Precision. Within-run precision was determined by assaying three pooled sera ten times each. The pools contained 146, 323 and 815 mg/dl triglyceride respectively.

Interaction. Experiments were designed to evaluate the percentage of a value obtained by the proposed method which could be attributed to "carryover" from the preceding sample. To derive the percentage interaction, three specimens of serum were analyzed in sequence. The first (N_1) and the third (N_3) contained 146 mg/dl triglyceride and the second contained 323 mg/dl of triglyceride. The percentage interaction was calculated from the formula:

$$\%I = \frac{N_3 - N_1}{N_2} \times 100$$

The reported interaction values are the averages of ten runs at each sampling rate.

Sampling Rates and Ratios. Most of the AutoAnalyzer II^R data presented in this report were collected using a sampling rate of 50 samples/hr, with a ratio of sampling time to wash time of 8:1. Sample cams of 60/hr, 9:1 and 100/hr, 2:1 were also used.

Steady State Color Development. If a sample is aspirated continuously on a continuous flow analyzer, a steady state

value will eventually be obtained. Various instrumental and methodological parameters can affect the percentage of steady state attained. Among the parameters are sampling rate, sample to wash ratio, tubing sizes, reagent composition and surface tension of the reagent. Using a two-minute aspiration, which is sufficient to reach steady state with the proposed method, steady state values of 146, 323 and 815 mg/dl were obtained on three serum pools. This allowed evaluation of various parameters and their effect on the percentage of steady state reached under those conditions.

Protein and Turbidity Effects. The protein effect was evaluated by leaving the colorimeter gain previously set for a triglyceride run unchanged, and running the samples as usual otherwise, except that no lipase was used in either channel. All traces of lipase in tubing were removed by a five minute treatment with 1 M NaOH. Turbidity effects were evaluated using the proposed method with the dye omitted from both sample and blank channels.

Specificity. Cholesterol production experiments were performed using the cholesterol detection reagent described by Allain et al. (1974). The experiments were done by adding serum to the cholesterol detection system, omitting cholesterol esterase. When endogenous cholesterol had reacted, an aliquot of lipase was added, and the reagent monitored for production of any additional cholesterol. Appropriate control

experiments demonstrated that the system was a valid way of measuring the presence of cholesterol esterase activity.

Experiments directed at the detection of phospholipase activity in lipase from Rhizopus arrhizus were conducted as follows: Extracts of serum were made by the method of Cham and Knowles (1976). The first, or "Blank" extract, was prepared by extraction of serum for 30 minutes with butanol-diisopropyl ether 6:4. This extract contained less than ten percent of preextraction levels of both triacylglycerols and phospholipids. This extract served as a blank allowing the determination of the effect of all non-lipid on the absorbance change of the fatty acid detection system. The second, or "Sample" extract, prepared by extraction of serum for 24 hours with diisopropyl ether, contained 25% and 90%, respectively, of pre-extraction levels of triacylglycerols and phospholipids. If phospholipase activity were present in the lipase, fatty acid production above that calculated from the amount of triacylglycerol present in the "Sample" extract would be seen when the extract was added as a serum sample to a reagent containing: BSP, 200 mg/l; BSA, 4.2 g/l; oleic acid, 0.08 mmol/l; sodium azide, 2 g/l and lipase from Rhizopus arrhizus, 2400 U/l. Control experiments in which the "Blank" extract was used, and also in which phospholipase, at a level of 2000 U/liter was used were performed, and appropriate corrections made. Triacylglycerol values were determined before and after extraction by the Calbiochem method. Phospholipid values were obtained before and after extraction by the method of Tietz (1976).

Methods Comparison. To evaluate the performance of the proposed method against commercially available methods for the determination of serum triglycerides, several groups of sera were analyzed. The sera for each group were chosen from all sera run over a period of several days at a local hospital. The sera were chosen to visually represent a large range of turbidity, from clear to grossly turbid. Each serum was then assayed, usually in duplicate, by the Calbiochem and Harleco methods, as well as the proposed method. Both commercial methods were used according to the manufacturer's instructions on a Gilford System 4 computer assisted analyzer. Both triacylglycerol and free glycerol values were obtained by the Calbiochem method, and the triacylglycerol values were corrected. The proposed method was run as described above. Each serum specimen was assayed in duplicate. After the first run, the order of samples was reversed so that any interaction effects would be averaged. Water and the sample used for standardization were run every tenth sample, which allowed monitoring of drift or other instrumental malfunctions.

Correlation and regression statistics were calculated and paired t-tests run to evaluate significance of differences in the values. The statistical evaluation was performed according to Westgard and Hunt (1973).

Stability Studies. Approximately 6 liters of reagent (excluding lipase) were prepared. Two liters were stored at each of three temperatures, 4°C, room temperature (approximately 25°C) and 37°C.

Lyophilized serum pools were prepared with assay values by the Calbiochem method of 113 and 537 mg/dl. On the initial day of the study, mixtures of the pools were assayed in duplicate, using one of the mixtures to standardize the proposed method. On succeeding weekly testing dates, duplicate determinations were run on each mixture of the serum pools for reagent stored at each temperature. The same mixtures were run in duplicate each testing date using freshly prepared reagent. The standardization was performed as described above. The criteria were as follows for continued stability:

a. No microbial growth or other visible sign of degradation may be present.

b. By use of adjustment of the colorimeter gain, it must be possible to obtain the assay values obtained on the first day of the stability study.

CHAPTER IV

RESULTS

A. Selection of Components of the Fatty Acid Detection Reagent

Albumin Choice. Protein fractions from a variety of species were screened for use in the proposed method. Table V shows that serum albumin from every source tested was acceptable. On the other hand, non-serum albumins such as lactalbumin and ovalbumin, as well as β- and γ-globulins were not acceptable. Bovine serum albumin (Fraction V) was chosen because of adequate response, low cost and ready availability. Albumin which had been defatted gave responses essentially identical to untreated albumin samples.

Dye Selection. Forty dyes, representing 14 chemical classes, were evaluated for response in the proposed method. Thirty-four dyes, representing ten chemical classes satisfied the criterion for minimum response set forth in the Methods section. The results of the evaluation are shown in Table VI. Sulfobromophthalein was chosen on the basis of its affinity of binding to BSA, and reversal of the binding by fatty acid. Difference scans using BSP are presented in Figures 1 and 2. The effect of the addition of fatty acid and serum, respectively, to the reagent are shown, and the results of serum hydrolysis are shown.

Table V

Responses of Dye-albumin Complexes Containing Sulfobromophthalein and Various Proteins to the Addition of Oleic Acid

Source of Protein	Change in absorbance at 580 nm upon addition of oleic acid[3]
Albumin, Fraction V[1]	
Rat	+0.424
Equine	+0.430
Canine	+0.828
Bovine	+0.450
Ovine	+0.426
Porcine	+0.590
Chicken	+0.286
Goat	+0.584
Human	+0.824
Rabbit	+0.484
Lactalbumin, Milk[2]	-0.120
Ovalbumin, Egg[2]	-0.050
β-Globulin, Fraction II[1], Bovine	-0.015
γ-Globulin, Fraction III[1], Bovine	+0.022

[1] Bovine serum fractions II, III and V prepared according to Cohn et al, JACS 68: 459-475 (1946) were used at a concentration of 3.45 mg/ml.

[2] Concentration 3.45 mg/ml

[3] Solutions contained sulfobromophthalein, 200 mg/l in 0.20 M sodium phosphate, pH 7.8. All proteins were at a final concentration of 3.45 mg/ml. Fifty µl of a 0.020 M solution of oleic acid in methanol were added to 3.00 mls of reagent.

Table VI

Responses Obtained with Various Dyes in the Albumin-dye Complex Reagent[1]

Dye	Concentration, M x 10^{-5}	Wavelength*, nm	Response, A
Azo Dyes			
Amaranthum	6.1	585	-0.12
Orange I	1.8	475	+0.11
Orange III	4.4	505	+0.22
		485	+0.23
Orange IV	9.3	470	+0.32
Congo Red	0.83	6.5	+0.17
Evans Blue	1.8	645	+0.04
Naphthol Blue Black	5.3	560	+0.21
		650	-0.17
HABA	2.8	340	+0.11
Ponceau 3R	3.2	495	+0.08
Ponceau S	3.3	500	+0.18
		565	-0.12
Azocarmine	5.9	490	+0.04
		570	-0.02
Sudan III	Not Soluble		
Sudan IV	Not Soluble		
Sulfonphthatein Dyes			
Bromocresol Green	1.5	615	+0.13
Bromocresol Purple	212	615	-0.17
		580	+0.16
Bromophenol Blue	1.7	585	+0.64
		625	-0.52
Bromothymol Blue	1.7	430	+0.04
		560	-0.03
Chlorophenol Red	1.7	570	+0.33
		600	-0.27
Cresol Red	3.1	570	+0.10
Metacresol Purple	8.4	435	+0.12
		585	+0.08
Sulfobromophthalein	25	580	+0.45
Thymol Blue	9.1	480	+0.11
		370	+0.03
		4.0	-0.01

Table VI - Continued

Triphenyl Methane Dyes			
Coomassie Brilliant Blue	3.7	550	+0.10
Crystal Violet	1.7	525 595	+0.10 -0.13
Malachite Green	1.7	620	+0.13
Fluorescein Dyes			
Dichlorofluorescein	0.83	500 520	+0.27 -0.42
Fluorescein	0.84	485 5.0	+0.16 -0.22
Fluorescein Isothiocyanate	1.8	485 510	+0.12 -0.16
Rhodamine 6 G	Produced a response with fatty acid in the absence of albumin.		
Phenoxazine Dyes			
Resazurin	45	585 630	+0.26 -0.50
Brilliant Cresyl Blue	10	600	+0.13
Nile Blue A	Produced a response with fatty acid in the absence of albumin		
Napthalene Sulfonic Acid Dyes			
Anilino Naphthalene Sulfonic acid	0.88	330 385	+0.09 -0.10
Naphthol Yellow S	9.4	430	+0.08
Indigo Dyes			
Indigo Carmine	9.0	600	+0.14
Acridine Dyes			
Acriflavine	44	470	+0.13
Anthroquinone Dyes			
Alizarin Red 5	10	450 350	+-.05 -0.09
Benzoquinone Dyes			
Dichloro indophenol	5.06	No Response	
Tricarbocyanine Dyes			
Indocyanine Green	4.4	800	+0.55
Copper Phtholocyanin Dyes			
Alcian Blue	Not Soluble		

* The wavelengths listed are those at which maximum absorbance changes occurred upon addition of oleic acid.

[1] The solutions contained 3.40 g/l BSA in 0.20 M sodium phosphate, pH 7.8. To this appropriate concentrations of dyes were added to yield a starting absorbance of less than one. These concentrations varied from 8.3×10^{-6} M to 4.5×10^{-4} M.

Figure 1. Difference spectra demonstrating the response of the proposed method to exogenous fatty acids. Difference scans of various combinations were made on a Hitachi 100 dual beam spectrophotometer. Reagent composition was 4.2 g/l BSA, 200 mg/l BSP, 2 g/l sodium azide, 15 ml/l FC-134 and 14 mmol/l oleic acid, all in 0.1 M sodium pyrophosphate, pH 8.0.

Curve A - dye albumin complex vs dye albumin complex
Curve B - dye albumin complex + 0.25 µmol oleic acid vs dye albumin complex
Curve C - dye albumin complex + 0.50 µmol oleic acid vs dye albumin complex

DIFFERENTIAL SPECTRAL SCANS

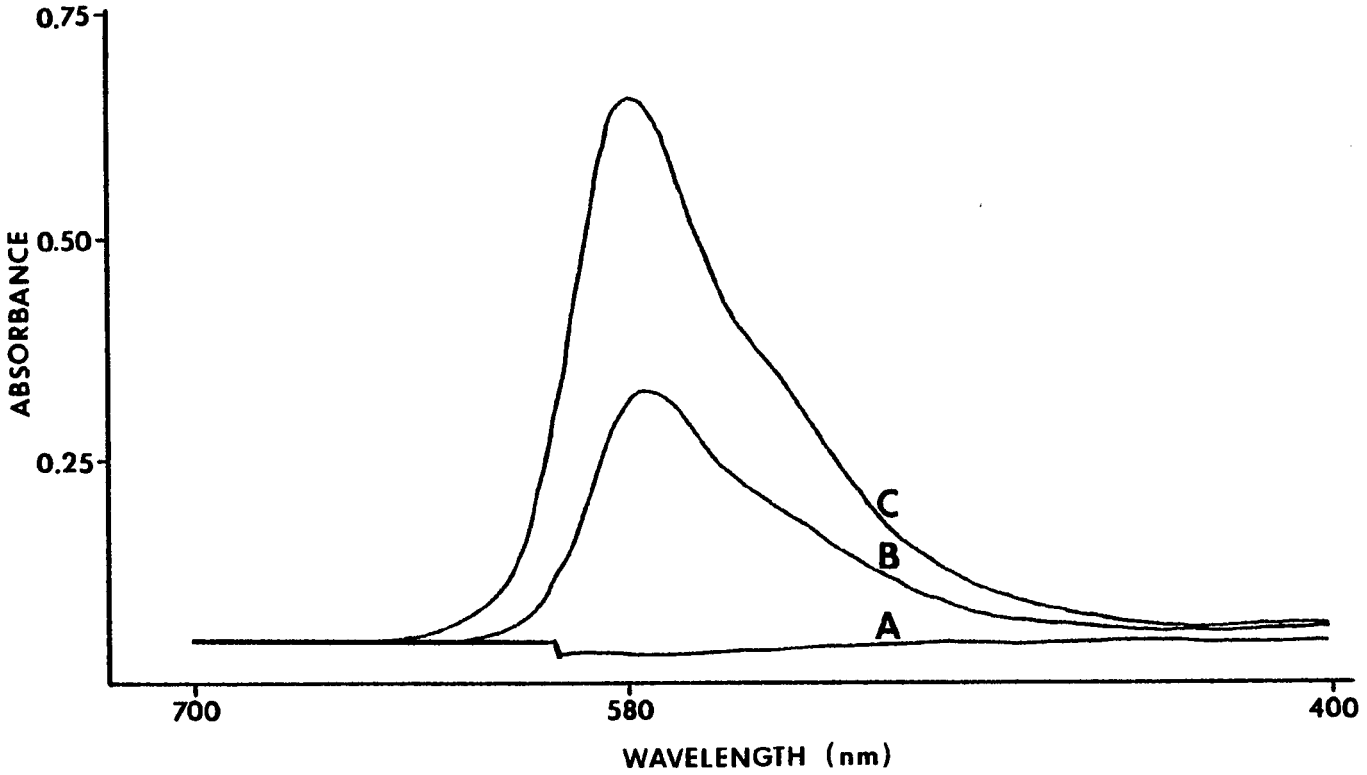


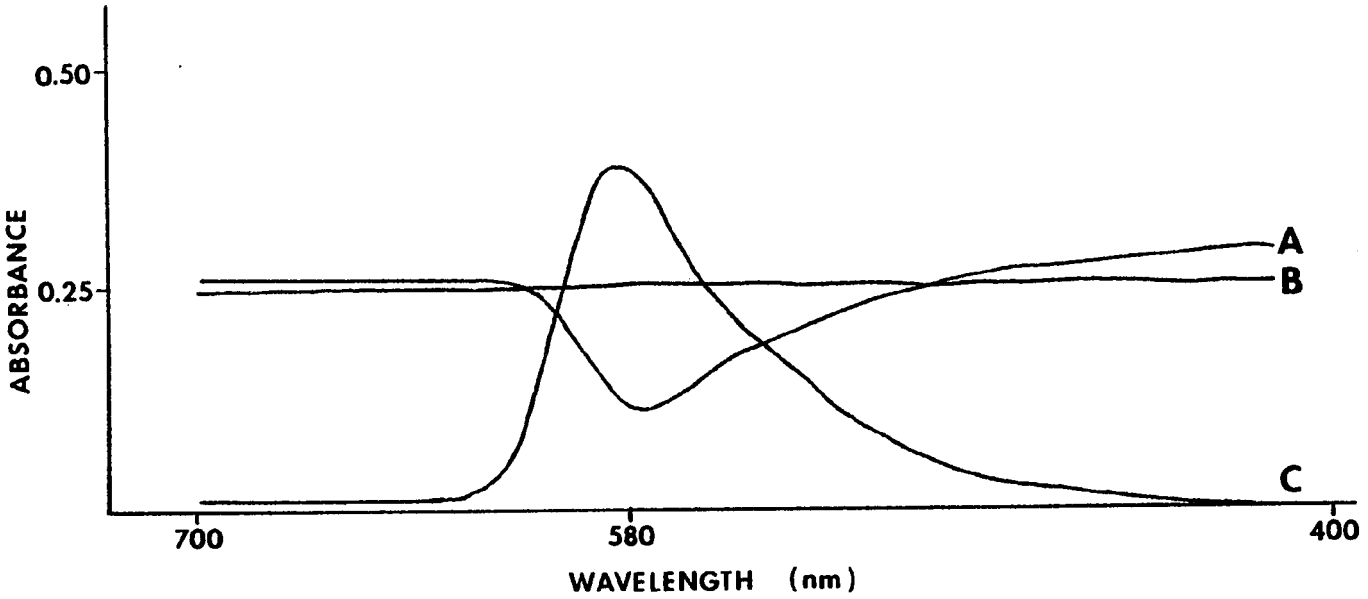
Figure 2. Difference spectra illustrating the protein effect and the response of the proposed method to fatty acids produced by lipase hydrolysis of serum triacylglycerols. Difference scans were performed on a Hitachi 100 dual beam spectrophotometer. The following combinations were scanned. The reagent from Figure 1 was used.

Curve A - Dye albumin complex + 50 µl serum vs dye albumin complex

Curve B - Dye albumin complex + 50 µl serum vs dye albumin complex vs 50 µl serum

Curve C - Dye albumin complex + 50 µl serum + 4800 U/ml lipase from Rhizopus arrhizus + 15 minutes vs dye albumin complex + 50 µl serum

DIFFERENTIAL SPECTRAL SCANS



Response to Fatty Acid. Once BSP and BSA were chosen, the extent of linearity of response of the proposed method to addition of increments of exogenous fatty acid was determined. As shown in curve A of Figure 3, the plot of response vs fatty acid added was decidedly alinear. However, as shown in curve B of Figure 3, addition of small amounts of fatty acid to the reagent resulted in a linear response up to 0.80 μmoles fatty acid added. This corresponds to a serum triacylglycerol level of 470 mg/dl. Oleic acid from 0.07 to 0.11 mmol/l and ricinoleic acid, from 0.14 to 0.29 mmol/l yielded acceptable linearity. The water solubility of ricinoleic acid was appealing, but subsequent experiments on the AutoAnalyzer II^{R} revealed that significantly higher response was obtained using oleic acid (Figure 4). The response of the proposed method to several naturally occurring fatty acids is shown in Figure 5.

Buffer Choice. The choice of BSP as the dye necessitated that the pH of the reagent be 7.6 or higher. Therefore buffers with pKa's in the range of 7.0 to 9.0 were examined. Sodium phosphate, sodium pyrophosphate, TRIS, glycylglycine, and bicine buffers were tried. The organic buffers all contained an amine moiety at various states of substitution. As shown in Figure 6, using bicine and glycylglycine as examples, the organic buffers did not yield asymptotic binding curves, while both phosphate and pyrophosphate did. Sodium phosphate was used in reagent preparations at pH 7.8, while sodium pyrophosphate was used at all higher pH levels.

Figure 3. The effect of addition of small amounts of fatty acid on the linearity of response to fatty acids of the proposed method.

Reagent compositions are: Curve A, 200 mg/l BSP and 3.45 g/l BSA in 0.2 M sodium phosphate, pH 7.8. Curve B, reagent for Curve A plus 0.14 μmoles of oleic acid per liter of reagent.

EFFECT OF ADDITION OF SMALL AMOUNTS OF FATTY ACID ON LINEARITY

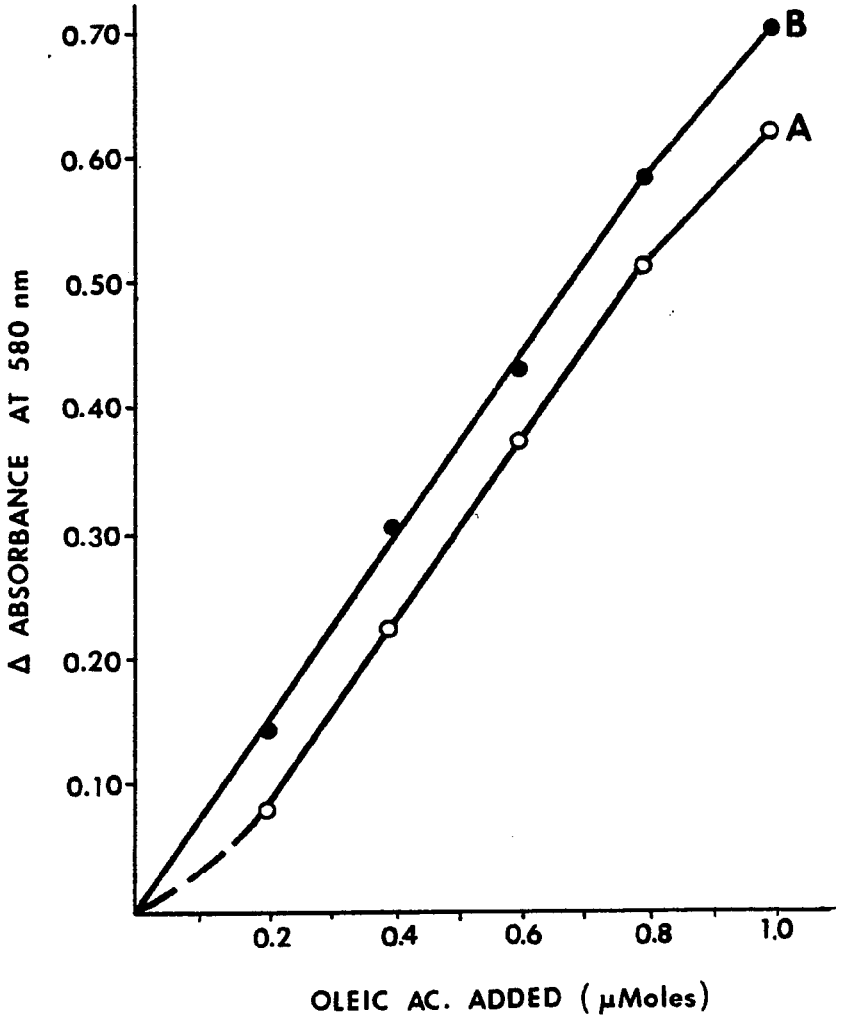


Figure 4. Comparison of oleic and ricinoleic acids for preadjustment of linearity of response to fatty acids of the proposed method.

reagent containing 4.2 g/l BSA, 200 mg/l BSP, 2 g/l sodium azide, 4800 U/l lipase and 15 ml/l FC-134. Two serum specimens with assay values of 145 and 740 mg/dl triacylglycerol were mixed to yield the intermediate values.

▲ = 0.10 µmol/ml of methanolic oleic acid.
□ = 0.14 µmol/ml of aqueous lithium ricinoleate.
o = 0.21 µmol/ml of aqueous lithium ricinoleate.
● = 0.28 µmol/ml of aqueous lithium ricinoleate.

EFFECT OF ADDITION OF FATTY ACID FOR PREADJUSTMENT OF LINEARITY

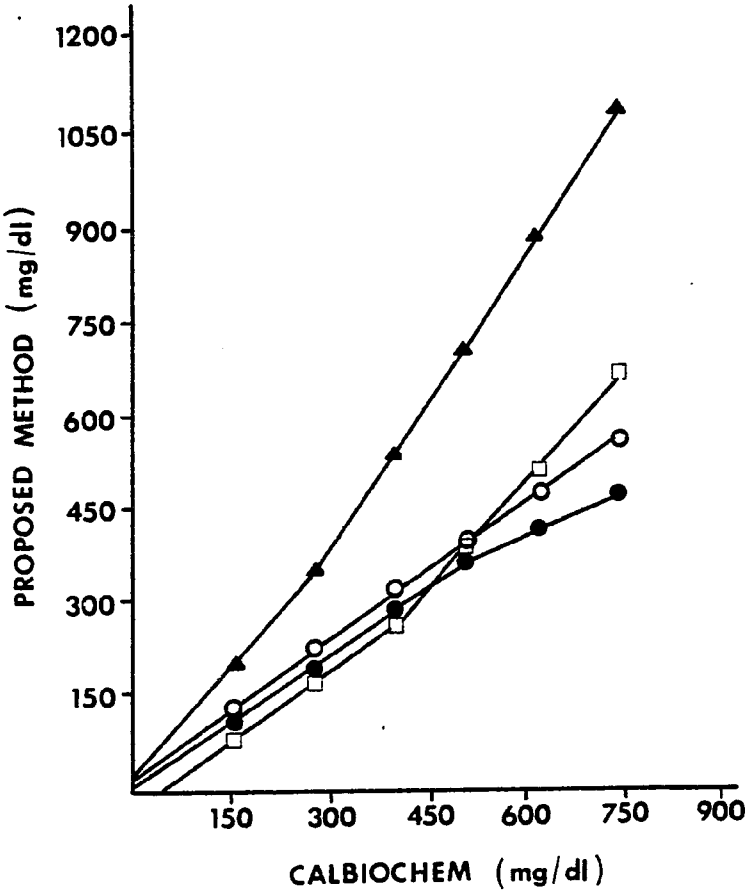


Figure 5. Response of the proposed method to several naturally occurring fatty acids.

Using the protocol described in the Methods section, and a reagent composed of 2.77 g/l BSA, 200 mg/l BSP and 0.04 μmoles/l oleic acid in 0.20 M sodium phosphate, pH 7.8, the response of the proposed method to several fatty acids was determined.

● = Oleic Acid
Δ = Myristic Acid
o = Lauric Acid
□ = Stearic Acid
▲ = Palmitic Acid

RESPONSE OF THE PROPOSED METHOD TO VARIOUS FATTY ACIDS

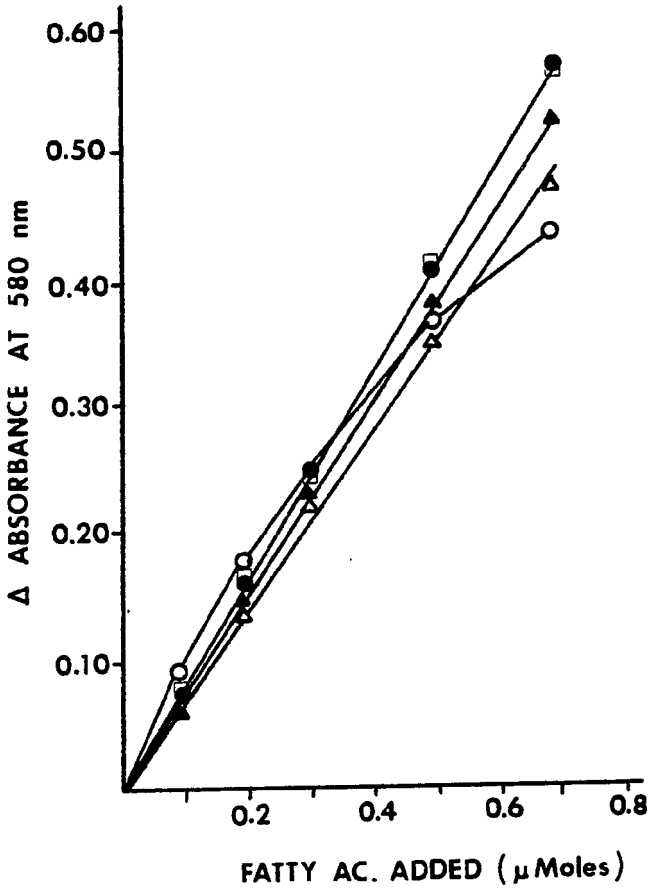
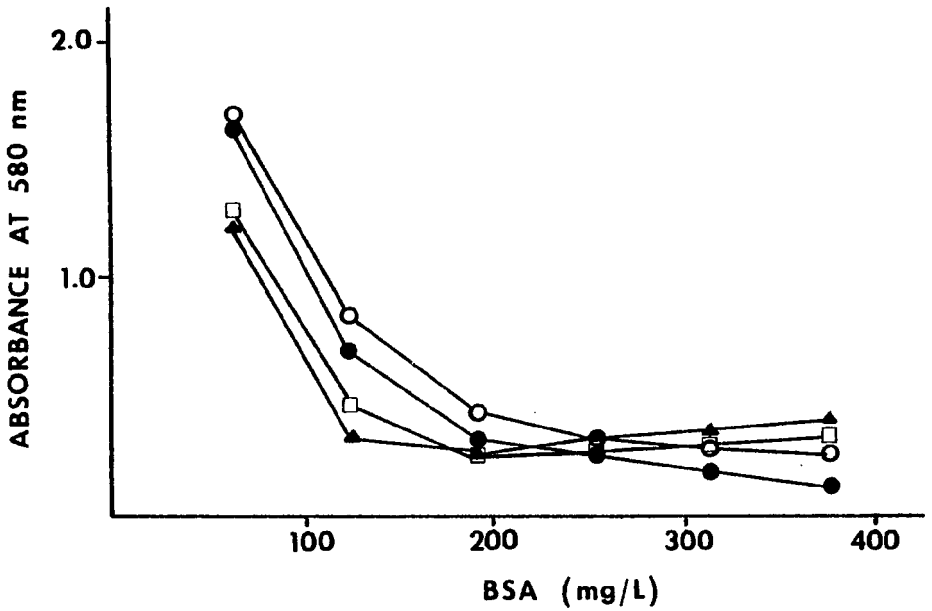


Figure 6. Binding of BSP to BSA at pH 8.5 in various buffers.

The curves were constructed by addition of 2.5 mls of BSP (100 mg/l) in the buffer noted to a cuvette. Various amounts of a 10 mg/ml solution of BSA in the same buffer were added to the cuvette. When necessary, buffer was added to yield a final volume of 4 mls.

▲ = Glycylglycine, 0.1 M
□ = Bicine, 0.1 M
o = Sodium pyrophosphate, 0.1 M
● = Sodium phosphate, 0.1 M

DYE - BINDING CURVES USING VARIOUS BUFFERS



Surfactant Selection. Surfactant evaluation (Table VII) was conducted using the protocol described in the Methods section. FC-134, a fluorinated alkyl quaternary ammonium iodide, was selected. The criteria for selection were excellent solubility, lack of interaction with components of the proposed reagent and excellent flow characteristics on the AutoAnalyzer II^{R}.

Factorial Experiment to Choose pH Level, Concentrations of Dye and Albumin and Level of Fatty Acid for Pre-adjustment. Using the AutoAnalyzer II^{R} manifold shown in Figure 7, the levels of pH, dye, albumin and fatty acid for preadjustment were varied over the ranges shown in Table VIII. Using trend analysis as described in the Methods section, a reagent with the following composition was chosen: 6.3 g/l BSA, 150 mg/l BSP, 0.375 mmol/l lithium ricinoleate, 2 g/l sodium azide and 15 ml/l FC-134 in 0.1 M sodium pyrophosphate pH 8.55. The plots of several combinations from this study which yielded the greatest response are shown in Figure 8.

B. Selection of Optimum Conditions and Concentrations for Assay of Serum Triacylglycerols

Lipase Selection. Lipases from six sources (Table IX) were considered, using the criteria of specificity, activity in the pH range 7.8 to 8.55 and cost. Lipase from Chromobacterium viscosum contained significant activity against cholesterol esters (Table X). Lipases from Geotrichium candidum, Candida cylindraceae and porcine pancreas were at least two orders of magnitude more costly than the other lipases considered. Lipase from Rhizopus delemar

Table VII

Surfactants Evaluated for Use in the Proposed Method[1]

Surfactant	Chemical Class	Supplier	Observed Response
Stradex PK-90	Polyphosphoric ester acid	Dexter Chemical	Turbidity upon addition
Zonyl FSA	Anionic Fluorosurfactant	Dupont	Depresses color yield
Zonyl FSB	Amphoteric Fluorosurfactant	Dupont	Turbidity upon addition
Zonyl FSC	Cationic Fluorosurfactant	Dupont	Turbidity upon addition
Zonyl FSJ	Anionic Fluorosurfactant	Dupont	Depresses color yield
Zonyl FSN	Nonionic Fluorosurfactant	Dupont	Depresses color yield
Zonyl FSP	Anionic Fluorosurfactant	Dupont	Poor Flow characteristics
Dow Corning 470A	Silicone Glycol Copolymer	Dow Corning	Depresses color yield
Dow Corning 190	Silicone Glycol Copolymer	Dow Corning	Turbidity upon addition
Dow Corning 193	Silicone Glycol Copolymer	Dow Corning	Depresses color yield
Aerosol Ay	Sodium diamyl sulfosuccinate	Cyanamid	Substrate for lipase
Triton X-100	octyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Triton X-305	octyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Triton X-705	octyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Triton N-101	nonyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Triton X-102	octyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Triton X-405	t-octyl phenoxy polyethoxy ethanol	Rohm & Haas	Depresses color yield
Brij-35	polyoxyethylene lauryl ether	Imperial Chemical Industries	Decreases color yield
Brij-96	polyoxyethylene oleyl ether	Imperial Chemical Industries	Decreases color yield
Tween 20	polyoxyethylene sorbitan monolaurate	Imperial Chemical Industries	Substrate for lipase
Tween 80	polyoxyethylene sorbitan monooleate	Imperial Chemical Industries	Substrate for lipase
FC-134	fluorinated alkyl quaternary ammonium iodide	Minnesotal Mining and Manufacturing	good flow characteristics, no interaction with reagent

[1] The surfactants listed above were evaluated according to the protocol in the Methods Section.

Figure 7. AutoAnalyzer manifold used to determine optimum concentrations and conditions for response of the proposed method to fatty acid.

The ranges of concentrations and conditions listed in Table VIII were evaluated, using lithium ricinoleate as a sample. The levels of lithium ricinoleate corresponded to serum triacylglycerol values from 300 to 1500 mg/dl in equal increments.

MANIFOLD DIAGRAM

OPTIMIZATION OF FATTY ACID DETECTION SYSTEM

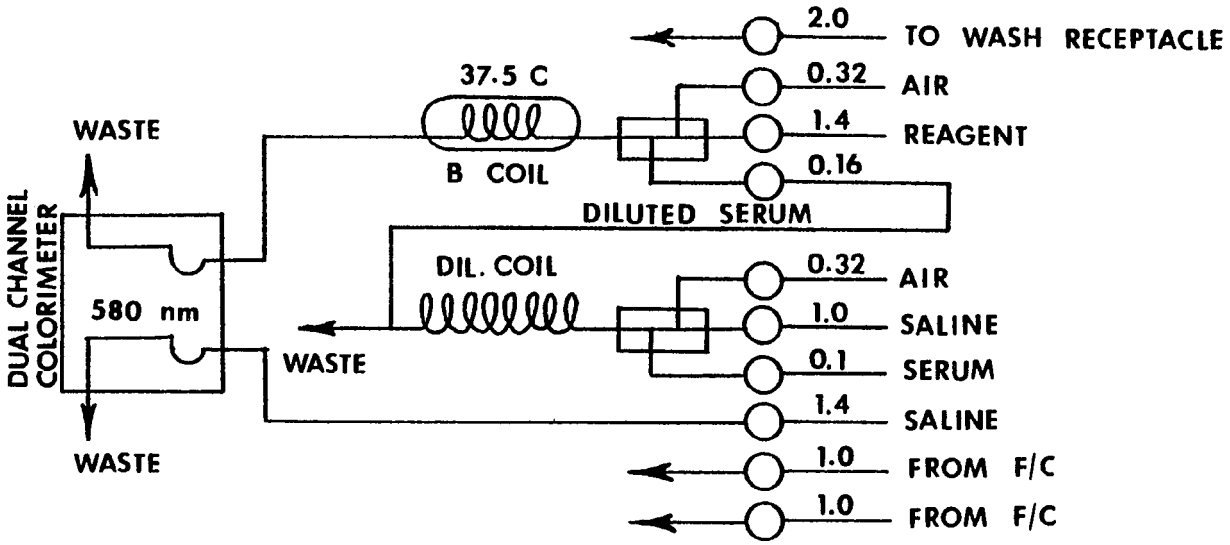


Table VIII

Ranges of Conditions and Concentrations Varied to Optimize Response of the Proposed Method to Fatty Acid[1]

pH[2]	8.35	8.45	8.55
BSP[2]	150 mg/l	200 mg/l	250 mg/l
BSA[2]	4.2 g/l	6.3 g/l	8.4 g/l
lithium ricinoleate[2] (for preadjustment)	0.15 mmol/l	0.25 mmol/l	0.35 mmol/l

[1] The components listed were added to 0.1 M sodium pyrophosphate containing 2 g/l sodium azide and 15 ml/l FC-134. The lithium ricinoleate levels listed are for an albumin level of 4.2 g/l and were raised proportionately at higher levels of albumin to yield a constant molar ratio of fatty acid to albumin.

[2] The conditions and concentrations chosen as optimum as a result of this study were: pH 8.55, BSP 150 mg/l, BSA 6.3 g/l, lithium ricinoleate 0.25 mmol/l.

Figure 8. Response of the proposed method at various combinations of pH, dye levels, albumin levels and levels of fatty acid for pre-adjustment.

Following trend analysis as described in the Methods section, the response of the proposed method to fatty acid at various combinations of the above parameters is plotted.

● = pH 8.55, 6.3 g/l BSA, 200 mg/l BSP, 0.25 mmol/l lithium ricinoleate
□ = pH 8.45, 8.4 g/l BSA, 200 mg/l BSP, 0.25 mmol/l lithium ricinoleate
▲ = pH 8.45, 6.3 g/l BSA, 150 mg/l BSP, 0.35 mmol/l lithium ricinoleate
o = pH 8.55, 6.3 g/l BSA, 200 mg/l BSP, 0.15 mmol/l lithium ricinoleate

RESPONSE OF THE FATTY ACID DETECTION SYSTEM TO VARIOUS REAGENT CONCENTRATIONS

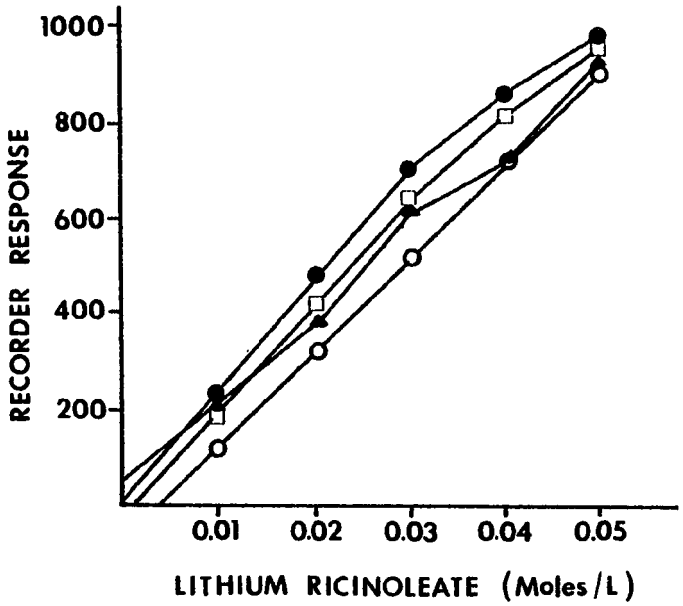


Table IX

Lipases Considered for the Proposed Method[1]

Lipase	pH[2] Optimum	Supplier	Specific[2] Activity	cost[2] cents/unit
Rhizopus delamar	5.6	Miles	600 u/mg	0.007
Geotrichium candidum	8.9	Sigma	12-25 u/mg	39.1
Candida cylindraceae	5.2 + 7.2	Worthington	500-800 u/mg	1.25
Porcine pancreas	8.2 to 9.2	Worthington	100-200 u/mg	1.4
Rhizopus arrhizus	3.5 + 7.5	Fermco	7000 u/mg	0.015
Chromobacterium viscosum	3 to 10	Toyo Jozo	1600 u/mg	1.25×10^{-4}

[1] The criteria of specificity, activity in the pH range 7.8 to 8.5 and cost were used to evaluate lipases from various sources for use in the proposed method.

[2] The information on these lipases was derived from the information provided by the supplier.

Table X

Evaluation of the Presence of Cholesterol Esterase Activity in Lipases from Two Sources[1]

	No addition	Lipase from* Rhizopus arrhizus	Lipase from** Chomobacterium viscosum	Cholesterol*** esterase
free cholesterol, mg/dl	45.6	45.1	37.4	45.1
cholesterol produced after addition, mg/dl	--	-0.5	95.1	100.0
Total Cholesterol, mg/dl	45.6	44.6	132.5	145.1

* 1000 U lipase/l

** 500 U lipase/l

*** 166 U/l of cholesterol esterase (pancreatic)

[1] Cholesterol production was measured using the protocol described in the methods section. The production of cholesterol above background levels upon addition of the lipase was considered indicative of the presence of cholesterol esterase activity in the lipase, as seen in the column labeled Lipase from Chomobacterium viscosum.

produced only 17% at pH 7.8 and 9% at pH 8.55 of the response expected if full hydrolysis of triacylglycerols had taken place.

Lipase from Rhizopus arrhizus was selected for further evaluation. Experiments directed at determination of the specificity of this lipase indicated no contamination by cholesterol esterase (Table X) or phospholipase (Table XI).

C. Adaptation of the Proposed Method to the AutoAnalyzer II^R for Use as a Serum Triacylglycerol Assay

Experiments at pH 7.8 and 8.55 (Table XII) had indicated total hydrolysis of triolein by lipase from Rhizopus arrhizus at both pH levels. Therefore, it was surprising when minimal response was obtained when serum triacylglycerols were hydrolyzed at pH 8.55 (Table XII). Additional experiments indicated that triolein was poorly hydrolyzed at this pH in the presence of serum (Table XII).

The response of the proposed method to fatty acid was approximately fourfold greater at pH 8.55 than at pH 8.0 (Table XIII). Therefore, a profile of the activity of lipase from Rhizopus arrhizus against serum triacylglycerols at various pH levels from 7.8 to 8.4 was obtained (Table XIII). This study revealed that the rate of hydrolysis of serum triacylglycerols declined rapidly above pH 8.0.

In an attempt to maintain the enhanced level of response to fatty acid obtained at pH 8.55 while allowing rapid hydrolysis of serum triacylglycerols, the AutoAnalyzer II^R manifold shown in

Table XI

Evaluation of Lipase From Rhizopus arrhizus for the Presence of Phospholipase Activity[1]

	Unextracted Serum	Extracted for 30 min with Butanol-isopropyl ether, 4:6	Extracted for 24 hours with isopropyl ether
Phospholipid Content, mg/dl	234	24	207
Triacylglycerol Content, mg/dl	119	11.6	28.5
Response (ΔA_{580}) in the Fatty Acid Dectection Reagent Upon Addition of:			
lipase from Rhizopus arrhizus*	--	-0.012	-0.007
phospholipase (bee venom)**	--	0.007 (9.4 mg/dl as Triolein)	0.042 56.5 mg/dl as Triolein)

* 2400 U/l of lipase added

** 2000 U/l phospholipase added

Oleic, 1.0 μmole, acid produced an absorbance change of 0.439

[1] Phospolipid levels were determined as described in the methods section. Triacylglycerol levels were determined using the Calbiochem method and correcting for free glycerol. The extractions were done according to Cham and Knowles (1976). The fatty acid detection reagent contained 200 mg/l BSP, 4.2 g/l BSA, 0.08 mmol/l oleic acid and 2 g/l sodium azide in 0.1 M sodium pyrophosphate, pH 8.0.

Table XII

Hydrolysis of Triolein by Lipase from *Rhizopus arrhizus* at pH 7.8 and at pH 8.55[1]

pH	Sample added	Fatty acid expected, μmol	Fatty acid recovered, μmol	% hydrolysis
7.8	serum, TG = 461 mg/dl	0.732	0.732	100.0
7.8	Triolein, 0.75 mg/ml	0.127	0.1233	97.1
7.8	" , 1.5 mg/ml	0.255	0.2604	102
7.8	" , 2.25 mg/ml	0.381	0.366	96.2
7.8	" , 3.0 mg/ml	0.508	0.513	101
8.55	" , 3.0 mg/ml	0.508	0.491	95.7
8.55	serum, TG = 576 mg/dl	0.984	0.008	0.8%
8.55	serum, TG = 296 mg/dl	0.506	0.011	2.27
8.55	serum[2] + triolein[3]	1.014	0.147	14.5%

[1] pH 7.8 experiments were performed in a reagent composed of 200 mg/l BSP, 4.2 g/l BSA, 0.14 mmol/l oleic acid, 1 g/l sodium azide and 1600 U/l of lipase in 0.2 M sodium phosphate. pH 8.55 experiments were performed using 150 mg/l BSP, 6.3 g/l BSA, 0.375 mmol/l lithium ricinoleate, 2 g/l sodium azide and 1600 U/l lipase in 0.1 M sodium pyrophosphate. Triolein was added in methanol.

[2] TG = 296 mg/dl.

[3] Triolein in methanol, 3 mg/ml.

Table XIII

Response of the Proposed Method at Various pH Levels[1]

pH	Response to 50 µl of 0.01 M Oleic Acid, mA	*Response to 50 µl of a serum with a 461 mg/dl Triacylglycerol Level	
		10 min (mA)	Max Response (mA)
7.8	0.105	0.164	0.164 (10 min)
8.0	0.218	0.196	0.351 (20 min)
8.2	0.435	0.143	0.662 (54 min)
8.4	0.833	0.016	0.900 (204 min)

* The lipase level in these studies was 532 U/ml

[1] Experiments were performed by adjusting the pH of a reagent of the composition: 200 mg/l BSP, 4.2 g/l BSA, 0.14 mmol/l lithium ricinoleate and 2 g/l sodium azide in 0.20 M sodium phosphate. To a 3 ml aliquot of this reagent, 1 µmol of oleic acid in methanol was added, and the response measured. To another aliquot, 50 µl of a serum with a triacylglycerol level of 461 mg/dl were added, lipase was added, and response measured at the times indicated.

Figure 9 was devised. In this configuration, the serum was hydrolyzed by the lipase at pH 7.8 in sodium phosphate buffer. The hydrolysate was then added to a color reagent at pH 8.55, the composition of which is shown in Table XIV. This approach was abandoned because of exceedingly poor correlation with serum triacylglycerol values obtained by the Calbiochem method, as shown in Table XV.

Based on the problems encountered using the previous approaches, the AutoAnalyzer II^{R} manifold shown in Figure 10 was devised. Using this manifold, variations of the levels of dye, albumin and fatty acid for preadjustment were performed at pH 8.0. The results of this study, along with the levels of components chosen as a result of the study, are shown in Table XVI.

The use of oleic acid rather than ricinoleic acid for preadjustment of response of the reagent to yield linear response to added fatty acid was studied. The results are shown in Figure 4 and indicate significantly greater response when oleic acid is used. A study to determine the optimum level of oleic acid was performed, and the results are shown in Table XVII. The level of oleic acid which yielded the regression line with the smallest intercept was chosen.

The optimum level of lipase in the proposed method was determined and a level of 4800 U/l was chosen (Table XVIII).

These studies resulted in the final reagent formulation shown in Table XIX, which was used in all further work.

Figure 9. AutoAnalyzer manifold with hydrolysis and color Measurement steps performed sequentially.

The reagent composition listed in Table XIV was used with this manifold in development of the proposed method.

MANIFOLD DIAGRAM

SEQUENTIAL HYDROLYSIS AND COLOR DEVELOPMENT

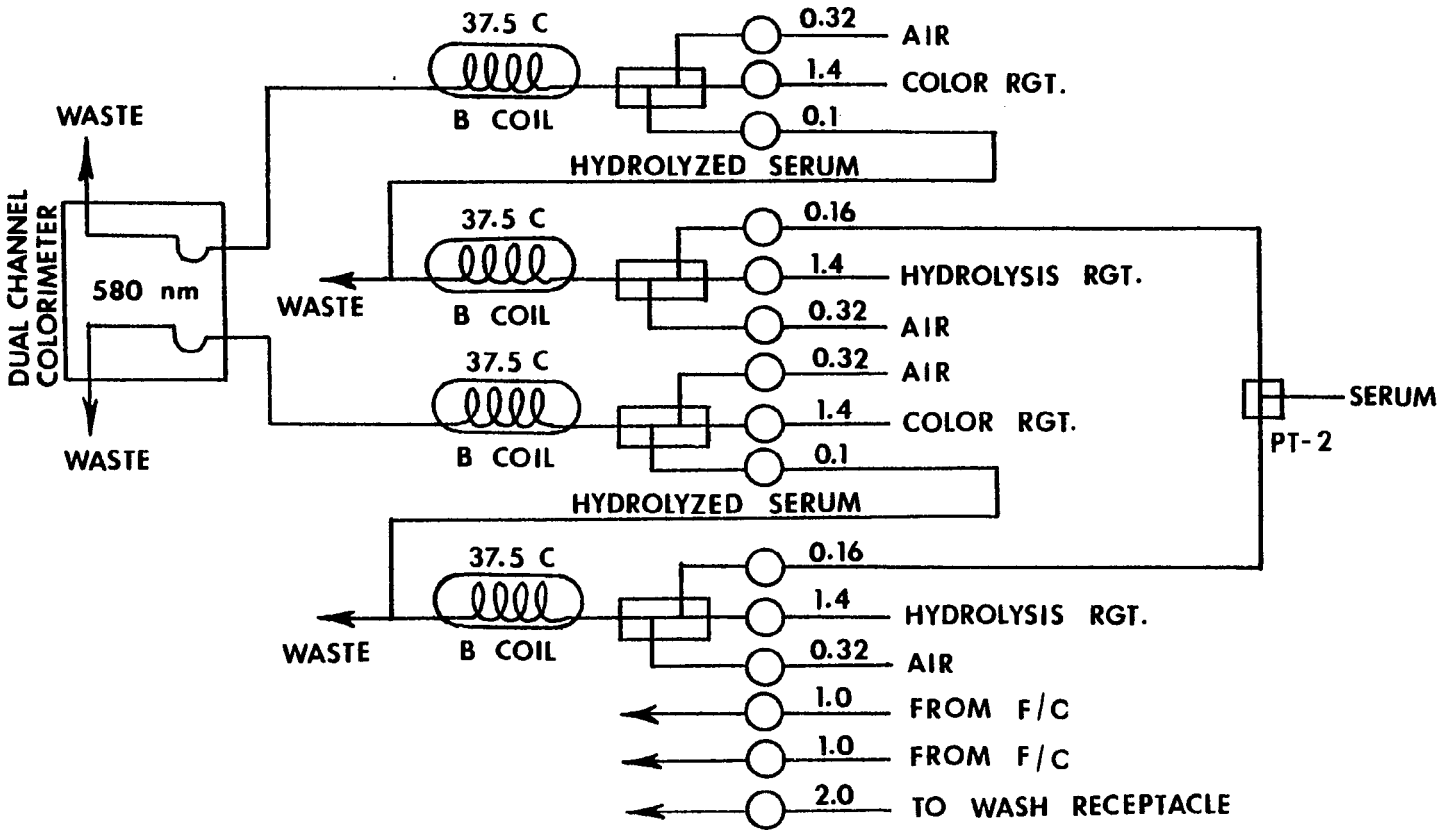


Table XIV

The Composition of Hydrolysis and Color Reagents Used When Hydrolysis and Color Development are Separated[1]

Hydrolysis buffer	0.05 M Sodium phosphate pH 7.8, 15 ml/l FC-134
	400 U/l lipase from Rhizopus arrhizus in sample channel, none in blank channel
Color Reagent	
Buffer	0.15 M sodium pyrophosphate, pH 8.55
BSP	150 mg/l
BSA	6.3 g/l
Lithium ricinoleate	0.25 mmol/l
Sodium azide	2 g/l
FC-134	15 ml/l

[1] Reagent compositions utilized in an attempt to combine hydrolysis at pH 7.8 and color measurement at pH 8.55, using the manifold shown in Figure 9.

Table XV

Aberrant Values Obtained When the Hydrolysis and Color Measurement Steps are Performed Sequentially[1]

Calbiochem Values, mg/dl	AA-II, pH 7.8[2] Hydrolysis in reagent	AA-II, pH 8.55[3] Hydrolysis in buffer alone
141	112	183
94	124	179
61	89	183
83	88	160
230	268	355
711	733	568
236	237	258
195	201	235
194	196	217
185	187	203
61	68	183
94	124	179
68	86	143

	Calbiochem vs proposed method, pH 7.8	Calbiochem vs proposed method, pH 8.55
r	0.995	0.959
slope	1.008	0.635
intercept	10.8	119.3

[1] The reagents described in Table XIV and the manifold in Fig. 9 were used to obtain the "pH 8.55" values. The manifold in Figure 10 was used to obtain the "pH 7.8" values, using a reagent composed of: BSA, 4.2 g/l; BSP, 200 mg/l; sodium azide, 2 g/l; lithium ricinoleate, 0.14 mmol/l, all in 0.20 M sodium phosphate, pH 7.8. Lipase levels for both runs were 1600 U/l.

[2] Hydrolysis took place in the total reagent, pH 7.8.

[3] Hydrolysis took place in 0.05 M buffer, pH 7.8, then the hydrolysate was added to color reagent at pH 8.55.

Figure 10. Auto Analyzer manifold diagram for the proposed Method.

This manifold was used for the data gathering portions of the proposed method.

MANIFOLD DIAGRAM
PROPOSED METHOD

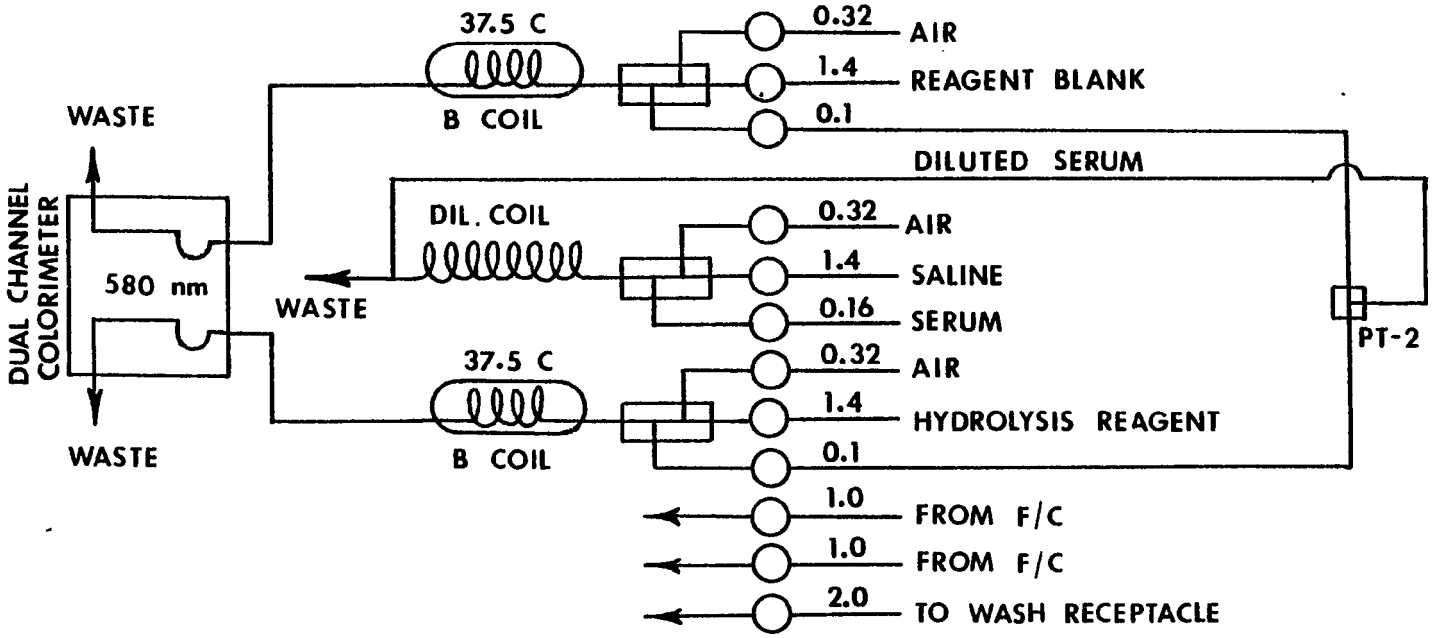


Table XVI

Variation of Levels of Dye, Albumin and Fatty Acid for Readjustment to Optimize the Response of the Proposed method[1]

Albumin, g/l	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	4.2	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4	8.4
Dye, mg/l	100	100	100	150	150	150	200*	200	200	100	100	100	150	150	150	200	200	200	100	100	100	150	150	150	200	200	200
Lithium Ricinoleate, mmol/l	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28	0.14	0.21	0.28
Calbiochem value, mg/dl	Found by AutoAnalyzer II® - mg/dl																										
145	46	71	58	93	120	95	152	161	170	--	--	--	--	16	20	20	27	39	6	16	29	18	35	53	44	70	103
271	76	119	95	168	209	161	244	261	261	--				28	**	35	40	65	9	28	47	41	55	108	72	105	157
388	112	179	144	253	299	230	368	369	369					47	53	50	69	102	18	47	75	65	78	171	118	176	223
505	156	230	190	311	405	310	510	560	460					65	80	72	99	147	26	65	105	93	102	237	175	259	313
611	209	287	230	472	500	368	657	612	525					90	97	93	130	185	39	90	142	134	129	310	211	334	405
740	260	399	262	582	592	434	783	709	606					137	138	131	175	247	61	137	191	178	163	386	334	415	474
r	0.993	0.987	0.997	0.986	0.999	0.999	0.996	0.990	0.999	--	--	--	--	0.972	--	0.984	0.987	0.993	0.962	0.972	0.987	0.988	0.995	0.998	0.972	0.991	0.994
M	0.367	0.531	0.357	0.829	0.811	0.582	1.10	0.968	0.750	--	--	--	--	0.197	--	0.183	0.253	0.348	0.091	0.197	0.273	0.269	0.215	0.568	0.468	0.606	0.653
b	-19.7	-21.9	4.75	-54.2	-5.49	8.14	-35.8	16.0	62.9	--	--	--	--	-23.4	--	-14.1	-22.0	-23.7	-13.7	-23.4	-23.0	-31.0	-1.79	-40.9	-48.6	-42.0	-10.1

* Levels of components chosen as a result of the study are: 4.2 g/l BSA, 200 mg/l RSP, 0.14 mmol/l lithium ricinoleate.

** Short Sample

[1] The autoanalyzer manifold shown in Figure 10 was used to conduct the variations. The levels of components shown were in 0.10 M sodium pyrophosphate, pH 8.0, containing 2 g/l sodium azide, 15 ml/l FC-134 and 3200 U/l lipase from *Rhizopus arrhizus*.

Table XVII

Variation of the Level of Oleic Acid Used to Adjust Linearity of Response of the Proposed Method[1]

Calbiochem Value, mg/dl	Oleic Acid Level, mmol/l					
	0.07	0.08*	0.09	0.10	0.11	0.12
110	117	124	138	135	141	142
203	193	201	220	211	216	219
293	274	286	306	292	295	296
379	362	372	386	372	374	373
457	448	453	483	454	455	447
538	546	555	565	538	539	520
r	0.997	0.998	0.999	0.999	0.999	0.970
m	1.15	1.09	1.00	0.944	0.931	0.865
b	-7.6	2.0	18.5	22.3	29.5	61.8

* The level chosen was 0.08 mmol/ml on the basis of the smallest y intercept.

[1] The manifold described in Figure 10 and a reagent of the composition: 4.2 g/l BSA, 200 mg/l BSP, 2 g/l sodium azide, 15 ml/l FC-134, and 3200 U/l lipase from Rhizopus arrhizus in 0.1 M sodium pyrophosphate, pH 8.0, were used to assay mixtures of two serum specimens at each level of oleic acid. Results were normalized for comparison.

Table XVIII

Variation of Lipase Levels to Establish an Adequate Level[1]

Serum #	Triacylglycerol Value @ 800 U/l (mg/dl) lipase	Triacylglycerol Value @ 1600 U/l (mg/dl) lipase	Triacylglycerol Value @ 3200 U/l (mg/dl) lipase	Triacylglycerol Value @ 6000 U/l (mg/dl) lipase
1	95	160	190	175
2	375	455	507	498
3	145	240	275	268
4	104	185	237	210
5	220	320	355	361
6	312	405	461	427
7	450	530	570	560
8	260	345	380	380
9	115	215	260	250
10	385	480	527	525
11	505	645	685	690
12	165	207	239	247
13	42	62	85	94
14	387	390	418	418
n	14	14	14	14
$\bar{x}$	254.3	331.4	370.6	364.5
$\bar{y}$	364.5	364.5	364.5	NA
t***	10.5	13.6	1.82	NA
P	<0.001	<0.001	$0.1 \geq p \geq 0.05$	
$\frac{\bar{x}}{\bar{y}}$**	0.652	0.888	1.018	
$SD_{(x/y)}$**	0.141	0.0728	0.0548	
$CV_{(x/y)}$(%)	21.6	8.21	5.48	

[1] Lipase levels were varied using the manifold described in Figure 10 and a reagent of the composition: 200 mg/l BSP, 4.2 g/l BSA, 0.08 mmol/l oleic acid and 2 g/l sodium azide in 0.10 M sodium pyrophosphate, pH 8.0.

** The mean and standard deviation of the ratio of each value to the value at 6000 U/l.

*** All "t" test values are paired to the 6000 U/l values.

Table XIX

Composition of the Proposed Reagent

	Sample Stream	Blank Stream
Buffer	0.1 M sodium pyrophosphate pH 8.0	0.1 M sodium pyrophosphate pH 8.0
BSP	200 mg/l	200 mg/l
Bovine serum albumin	4.2 g/l	4.2 g/l
Oleic acid	0.08 mmol/l	0.08 mmol/l
Sodium azide	2 g/l	2 g/l
FC-134	15 ml/l	15 ml/l
Lipase (Rhizopus arrhizus)	4800 U/l	---

Standardization. Standardization of the proposed method had in the early stages been accomplished using lyophilized samples which were assayed by the Calbiochem method. Certain studies (Table XX) indicated that serum specimens yielded, on the average, lower values by the proposed method after lyophilization than prior to lyophilization.

The results of a study, presented in Table XXI, indicate that either liquid or frozen serum specimens are acceptable for standardization, while lyophilized specimens are not.

D. Evaluation of the Proposed Method

Precision. Precision was evaluated at three serum triacylglycerol levels. Excellent within run precision was obtained, even at high sampling rates, as shown in Table XXII.

Linearity and Recovery. The proposed method is linear to at least 500 mg/dl triacylglycerol, as shown in Figure 11. Recovery values ranged from 96 to 101% over the range of usefulness of the method. Recovery values are listed in Table XXIII.

Interaction. Replicate determinations of levels of interaction yielded values of 1.27% at a sampling rate of 50 per hour, 8:1; and a value of 2.22% at 100 per hour, 2:1.

Steady State Color Development. The percent of steady state color development for the proposed method was evaluated at two sampling rates. The results are shown in Table XXIV. A recorder tracing (Figure 12) shows the flat tops on the peaks, indicative of near-steady state operation.

Table XX

Effect of Lyophilization of Serum on Values Obtained by the Proposed Method[1]

Specimen	Value by Proposed Method Prior to Lyophilization	Value by Proposed Method After Lyophilization	% Difference
1	83	86	+3.4
2	88	60	-31.8
3	131	125	-4.8
4	167	143	-14.4
5	124	98	-21.0
6	205	170	-17.1
7	130	90	-30.8
8	151	120	-20.5
9	125	86	-31.2
10	268	212	-20.9
11	107	86	-19.6
12	180	140	-17.6
Mean	146	118	

Average percent difference = -18.9%

$t = 6.25$
$p \leqq 0.001$

[1] The serum specimens were assayed in both the pre- and post-lyophilized states using the reagent described in Table XIX and the manifold shown in Figure 10.

Table XXI

The Effect of Various Serum Treatments on the Standardization of the Proposed Method[1]

	Calbiochem vs Pool 1 Liquid	Calbiochem vs Pool 1 Frozen	Calbiochem vs Pool 1 Lyophilized	Calbiochem vs Pool 2 Liquid	Calbiochem vs Pool 2 Frozen	Calbiochem vs Pool 2 Lyophilized
n	24	24	23*	24	24	24
r	0.963	0.984	0.975	0.979	0.978	0.981
m	0.90	1.06	1.76	0.96	1.02	1.22
b	23.8	13.3	-15.0	17.2	21.0	20.4
$\bar{x}$	230.5	230.5	206.3	230.5	230.5	230.5
$\bar{y}$	231.0	261.9	347.7	251.6	255.1	301.5
t	0.46	-3.86	7.25	-1.89	-3.35	-6.40
	$(0.9 \geq p \geq 0.5)$	$p<0.001$	$p<0.001$	$(0.1 \geq p \geq 0.05)$	$(0.01 \geq p \geq 0.005)$	$p<0.001$

* One sample was off scale.

[1] The reagent from Table XIX and the manifold from Figure 10 were used to assay 24 serum specimens. The Calbiochem values obtained on each serum treatment, corrected for free glycerol, were used to set the colorimeter gain.

Table XXII

Within Run Precision Obtained Using the Proposed Method[1]

Sample Number	50/hr, 8:1 Sampling Rate				100/hr, 2:1 Sampling Rate			
	N	Mean	S.D.	C.V. (%)	N	Mean	S.D.	C.V. (%)
1	10	139.0	2.83	2.03	10	122.2	2.94	2.40
2	10	314.8	2.20	0.70	10	281.1	6.97	2.48
3	10	804.5	7.75	0.96	10	713.0	14.0	1.96

[1] Using the reagent from Table XIX and the manifold shown in Figure 10, ten replicate determinations were run on each of the serum pools at each of two sampling rates.

Figure 11. Linearity of the Proposed Method.

Quadruplicate determinations were made on mixtures of two serum pools with values of 140 and 575 mg/dl. The reagent from Table XIX and the manifold from Figure 10 were used.

PROPOSED METHOD LINEARITY

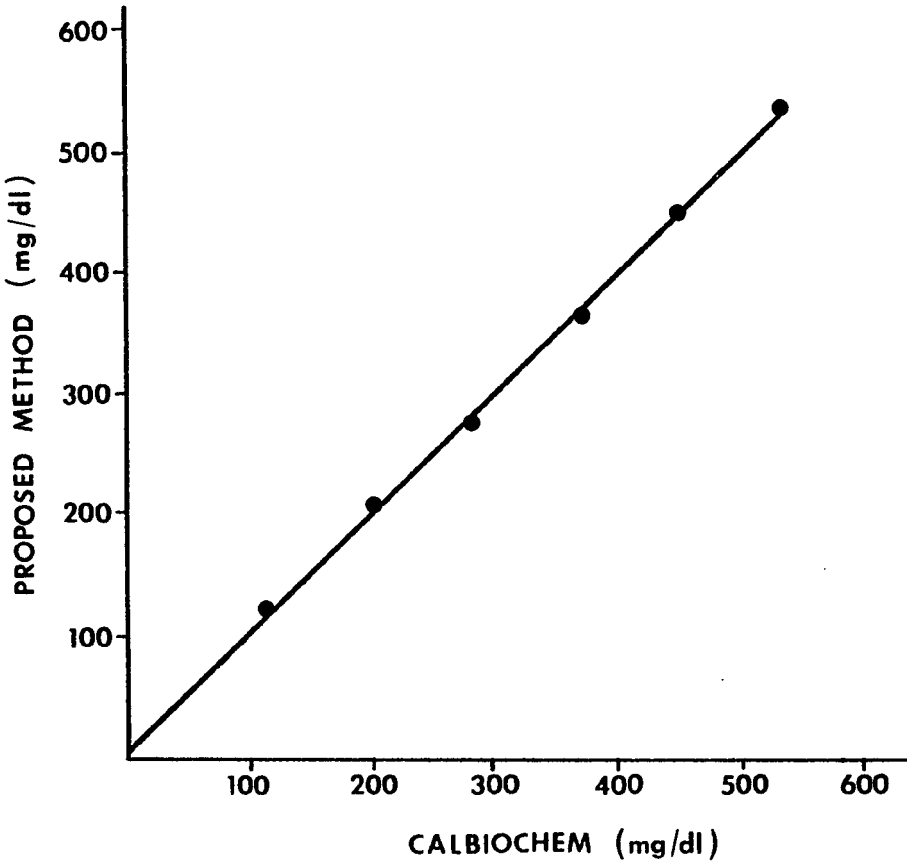


Table XXIII

Recovery of Serum Triacylglycerols Using the Proposed Method[1]

Calbiochem Value, mg/dl	Value Obtained Using Proposed Method, mg/dl	Value Expected Using Proposed Method, mg/dl	% Recovery
113	122	---	---
198	207	205	101
283	276	288	96
367	360	370	97
452	447	453	99
537	536	---	---

[1] Two serum pools were assayed by the proposed method. The expected values for mixtures of the pools were calculated and recoveries determined by comparing the values obtained by the proposed method on the mixtures. The conditions of Table XIX were used. All assays were performed in triplicate.

Table XXIV

Percent of Steady State Color Development
at Two Different Sampling Rates[1]

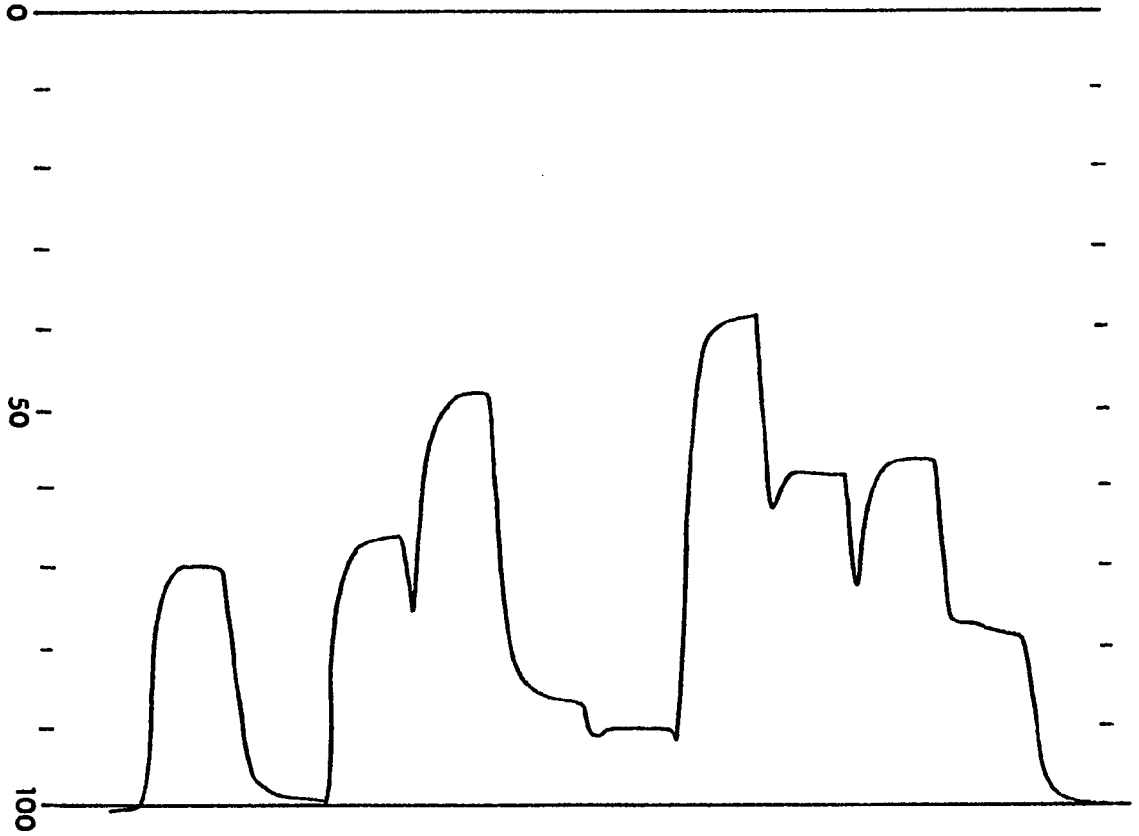
Serum Number	Steady State value, mg/dl	Mean Value at 50/hr, 8:1, mg/dl	Mean Value at 100/hr, 2:1, mg/dl
1	146	139 (95%)	122 (84%)
2	323	315 (97%)	281 (87%)
3	815	805 (99%)	713 (88%)

[1] The conditions of Table XIX were used to obtain steady state values on three serum pools. Each level was determined in triplicate.

Figure 12. Recorder tracing for the proposed method.

Random fresh serum samples were analyzed using the reagent listed in Table XIX and the manifold shown in Figure 10.

TYPICAL RECORDER TRACING



Protein and Turbidity Effects. The magnitude of the protein effect was determined on 53 serum specimens with an average serum triacylglycerol value of 175 mg/dl by the proposed method. The average protein effect was equivalent to an error of +10.3 mg/dl triacylglycerol. The turbidity effect was determined on the same 53 samples. This effect was equivalent to an average error of -1.4 mg/dl.

Methods Comparison. Fifty-three serum specimens, ranging in triacylglycerol content from 22 to 1108 mg/dl, were assayed by the Calbiochem method, the Harleco method and the proposed method. Statistical treatment of the data is given in Table XXV, and scatter-grams are given in Figures 13, 14 and 15.

Specificity. The specificity of the lipase used in this method was critical due to the lack of specificity in the detection reaction. For this reason, experiments were designed to determine the specificity of the lipase from Rhizopus arrhizus toward cholesterol esters and phospholipids. The cholesterol ester experiments were performed by removal of endogenous cholesterol from a serum specimen by use of cholesterol oxidase. The lipase was then added. Production of any additional cholesterol was taken as an indication of cholesterol esterase activity in the lipase. The cholesterol detection system of Allain et al. (1974), minus cholesterol esterase, was used. As can be seen from Table X no cholesterol esterase activity was detectable.

The phospholipid experiment was performed by making two extracts of a serum pool by the method of Cham and Knowles, as

Table XXV

Statistical Comparison of the Triacylglycerol Assay Results on 54 Serum Specimens by the Proposed Method and Two Commercially Available Serum Triacylglycerol Methods[1]

	Calbiochem vs Proposed	Harleco vs Proposed	Calbiochem vs Harleco
N	53	53	53
M	0.993	0.988	0.852
b	10.3	13.5	14.2
Bias	9.0	6.0	15.0
t	4.03 ($p=<0.001$)	0.99 ($0.4 \geq p \geq 0.2$)	2.38 ($0.5>P>0.02$)
Sy	9.63	23.4	2.62
SD_d	16.6	37.9	37.8
r	0.994	0.929	0.976

[1] The details of the serum assays are given in the methods section. The data treatment is according to Westgard and Hunt (1973).

Figure 13. Scattergram of a comparison between the Calbiochem method and the proposed method.

The reagents from Table XIX and the manifold from Figure 10 were used to assay 54 serum specimens. The Calbiochem method was run according to the manufacturer's instructions, and correction for free glycerol was included.

METHODS COMPARISON

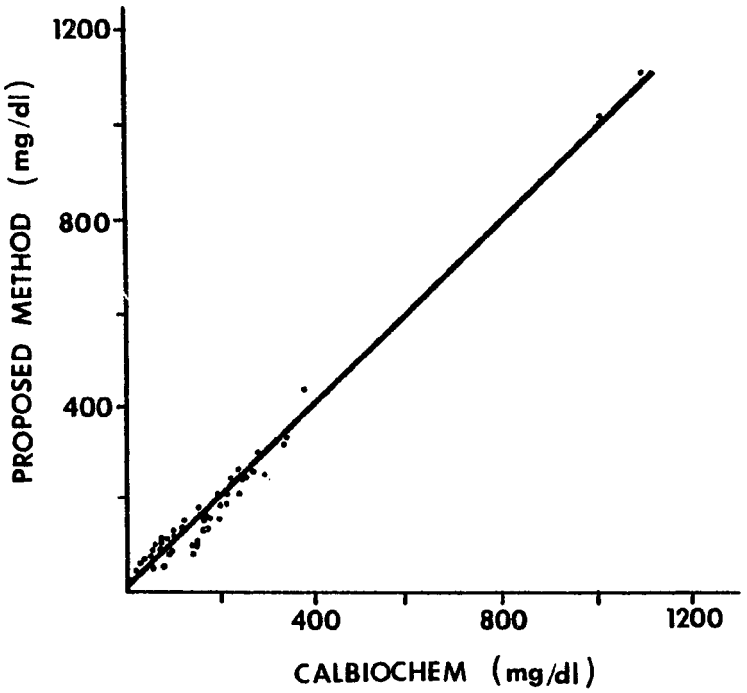


Figure 14. Scattergram for a comparison between the Harleco method and the proposed method.

The reagents from Table XIX and the manifold from Figure 10 were used to assay 54 serum specimens. The Harleco method was used according to the manufacturer's instructions. Samples containing endogeneous glycerol levels of 20 mg/dl or greater are indicated by a ◘ .

METHODS COMPARISON

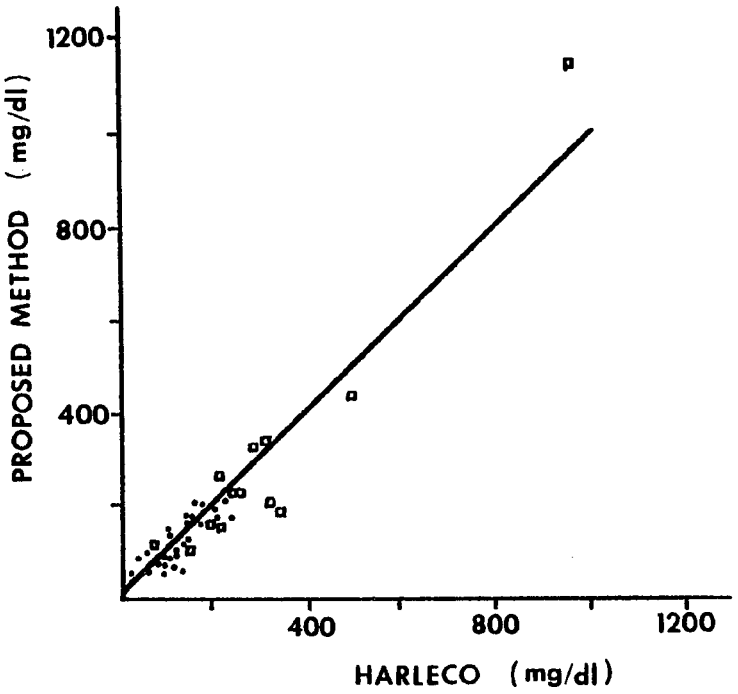
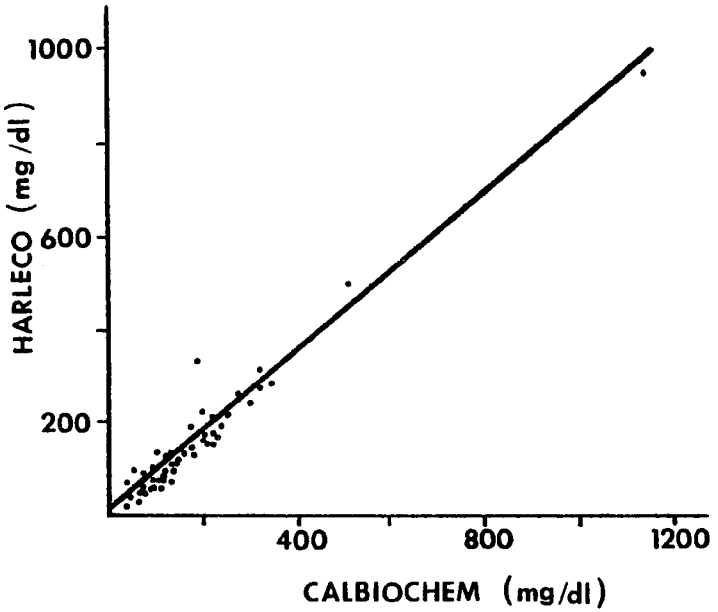


Figure 15. Scattergram for a comparison between the Calbiochem and Harleco methods.

Each method was used according to the manufacturer's instructions. No corrections for glycerol are included.

METHODS COMPARISON



described in the Methods section. Fatty acid levels in the serum extracts were determined before and after incubation with lipase. Since the serum extracts contained minimal amounts of triacylglycerols, it was possible, by use of appropriate blanks, to determine if any phospholipase activity was present. As can be seen from Table XI, no phospholipase activity was detectable.

Stability. Using the criteria outlined in the Methods section, the proposed reagent has been stable for three weeks at 37°C, and seven weeks at room temperature or at 4°C. A summary of the stability data is shown in Table XXVI.

Interferences. Addition of sodium salicylate, at a level of 20 mg/dl, to a group of lyophilized serum specimens did not result in any detectable change in assay values for triacylglycerols.

Table XXVI

Stability Data for the Proposed Method[1]

Day 0 Calbiochem Values mg/dl	1 Week TG value, mg/dl					2 Weeks TG value, mg/dl				3 Weeks TG value, mg/dl				5 Weeks TG value, mg/dl				7 Weeks TG value, mg/dl			
		4°	Room Temp.	37°	Fresh	4°	Room Temp.	37°	Fresh	4°	Room Temp.	37°	Fresh	4°	Room Temp.	37°	Fresh	4°	Room Temp.	37°	Fresh
113	122	122	130	131	125	127	125	131	131	130	127	118	129	118	129	126	130	130	103	128	133
198	207	217	210	217	194	205	193	200	200	211	210	214	206	200	198	211	204	187	185	191	185
283	276	282	289	281	280	280	269	276	280	269	278	275	277	270	279	260	276	290	298	300	280
367	360	387	377	372	352	366	353	361	363	359	371	365	368	380	360	352	369	377	401	385	354
452	447	480	472	461	448	454	437	460	457	450	462	454	458	449	449	425	445	478	520	446	430
537	536	583	515	550	544	558	527	556	540	539	551	542	546	598	541	514	548	572	633	568	560
Colorimeter Gain	418	440	460	440	409	452	452	500	424	430	430	505	435	460	445	515	455	500	580	780	495

[1] All data were gathered using the reagents from Table XIX, the manifold in Figure 10 and the protocol described in the methods section.

CHAPTER V

DISCUSSION

A considerable body of literature exists concerning the triacylglycerol content of serum, as documented in this dissertation. The level of sophistication of the assays has increased rapidly, resulting in several totally enzymatic methods. Two of the enzymatic methods are adaptable to continuous-flow automation. Unfortunately, the advances have also resulted in substantial increases in the cost of the assays, due to the complex enzymatic coupling systems. The International Marketing Service Survey (1978) estimated that 2.5×10^7 serum triacylglycerol assays were performed in the United States in 1977. This volume is so large that any successful new method must be adaptable to automation. Thus, the two primary concerns in the development of the proposed method were adaptability to automation and cost.

As can be seen from Tables I, II and III, the glycerol moiety of triacylglycerols has been exploited almost universally in the measurement of serum triacylglycerol levels. The insolubility of fatty acids in aqueous solutions, coupled with the wide distribution of fatty acids among serum lipids, have posed formidable problems to this mode of measurement. However, the use of a dye-albumin complex and a lipase confers a specificity to the proposed

method that allows aqueous determination of the fatty acids released from serum triacylglycerols by enzymatic hydrolysis.

Klotz (1946), in his early work concerning the interactions of dyes with albumin, laid the groundwork for the proposed method. The demonstration of the spectral changes associated with this interaction and the partial reversibility of these changes upon addition of competing ligands were fundamental to the proposed method. Finally, the demonstration, by Kucerova (1966), that fatty acids compete with sulfobromophthalein for binding sites on bovine serum albumin, while neither bilirubin or salts of bile acids do, indicated a significant degree of specificity.

The results of the evaluation of various protein sources for the proposed method were not surprising. All of the serum albumins which were tested yielded a significant response in the proposed method (Table V). On the other hand, lactalbumin, ovalbumin, β-globulin and γ-globulin yielded minimal response. Bovine serum albumin was chosen because of ready availability, adequate response and low cost.

The evaluation of dyes for suitability in the proposed method yielded results that were surprising. Several dyes were insoluble under the conditions of evaluation, and two dyes (Nile Blue A and Rhodamine 6G), which are used normally for fat staining, yielded a response to fatty acids whether or not albumin was present. With these exceptions, however, every dye tested was displaced from albumin by addition of fatty acid in quantities sufficient to be

spectrophotometrically detectable. These dyes included azo, sulfonphthalein, phenoxazine, naphthalene sulfonic acid, indigo, acridine, benzoquinone, tricarbocyanine and copper phthalocyanin dyes.

The difference spectra in Figures 1 and 2 demonstrate the following:

1. Addition of fatty acid to the dye-albumin complex produces a detectable spectral change.
2. The addition of a serum specimen to the dye-albumin complex produces a spectral change (the protein effect) in the opposite direction from the fatty acid spectral change. The protein effect may be compensated for by means of a blank.
3. Addition of lipase to a serum sample in the presence of the dye-albumin complex produces a spectral change of the same type and direction as the change produced by addition of fatty acid.

One of the keys to the usefulness of the proposed method as a serum triacylglycerol assay method was the finding, shown in Figure 5, that several naturally occurring fatty acids yielded essentially equal responses. If this had not been the case, the proposed method would have been useless, due to the great variety of fatty acids which occur in serum triacylglycerols.

A finding which is fundamental to the understanding of the nature of the competition between dye and fatty acid molecules for

binding sites on albumin is illustrated in Figure 3. When increments of exogenous fatty acid are added to the dye-albumin complex, and the spectral response plotted vs amount of fatty acid added, a curve with sigmoidal character is obtained. However, if a small amount of fatty acid is first added to the dye-albumin complex, the plot of response vs fatty acid added is linearly proportional over a useful range of fatty acid concentrations. There are several possible explanations for this finding. Spector (1975) has demonstrated that when fatty acid is added to a solution containing albumin and methyl orange, three moles of fatty acid must be added per mole of albumin before any dye is displaced. Spector also indicated that sites may exist on albumin which bind fatty acid but not dye. If this were the case, these sites would have to be saturated with fatty acid before any dye would be displaced. The addition of small amounts of fatty acid for pre-adjustment of the linearity of the dye-albumin complex apparently saturates binding sites on the albumin to which dye is then not bound. With proper pre-adjustment, additional increments of fatty acid displace dye from binding sites with affinities for both dye and fatty acid.

The influence of the buffer in which the dye-albumin complex is formed is demonstrated in Figure 6. When binding curves were produced in either sodium phosphate or sodium pyrophosphate the absorbance of the solutions decreased or remained constant below a certain dye to albumin ratio. When amine buffers, such as tris-hydroxymethyl aminomethane, glycylglycine or bicine were used, the absorbance of solutions would increase as dye to albumin ratios

were decreased. This could possibly be due to competition of the amino groups on the buffer with the dye for binding sites on the albumin (Spector, 1975).

The experiments directed at choice of optimum concentrations and conditions for response of the proposed method to exogenous fatty acid led to one of the most puzzling findings obtained during this research. As can be seen from the data presented in Tables XII and XIII, triolein is hydrolyzed quantitatively at pH 7.8 and pH 8.55 by lipase from Rhizopus arrhizus. This was an unexpected finding, since Bucolo and David (1973) and Wahlfeld, Klose and Munz (1975) had indicated that an esterase was necessary to achieve total hydrolysis of triacylglycerols using lipases from Rhizopus delemar and Rhizopus arrhizus, respectively. It is hypothesized that the high concentration of albumin present in the proposed reagent may act as a "sink," removing the fatty acids from solution and allowing the reaction to go to completion. Private communications with Fermco Biochemics, the supplier of the lipase used in the proposed method, support this hypothesis. At the same time, hydrolysis of serum triacylglycerols takes place slowly above pH 8.0 (Table XII). Even more curiously, the hydrolysis of triolein at pH 8.55 by lipase from Rhizopus arrhizus proceeds only about 25% to completion when serum is present. Two alternate hypotheses are advanced to account for these findings. It is possible, as the pH is raised, that the affinity of certain serum lipoproteins for serum triacylglycerols is increased, rendering the triacylglycerols less accessible to the lipase. Alternately, an inhibitor of the

lipase may be present in serum, manifesting its action more strongly as the pH is raised. The results of the research presented in this dissertation are not sufficient to choose between these alternate hypotheses. It is unfortunate that this phenomenon exists, since the response of the proposed method to fatty acid increases significantly with pH, as shown in Table XIII.

In Table XVIII, it can be seen that the use of a paired t-test is not useful in choosing an adequate level of lipase. If the triacylglycerol levels obtained at each of the other lipase levels are compared to the 6000 U/l lipase level, all of the other sets of values are significantly different, as measured by the t-test. On the other hand, it is apparent by inspection that significant differences exist between the values obtained at 800 and 1600 U/l lipase and those obtained at 6000 U/l lipase, while little difference is seen at the 3200 U/l and 6000 U/l levels. Thus, another approach was attempted. Each triacylglycerol value obtained at each of the other lipase levels was divided by the corresponding value at 6000 U/l lipase. The mean and standard deviation of the ratios at each level were calculated, along with the coefficient of variation. If perfect agreement were obtained between the triacylglycerol values obtained at any two levels of lipase, a mean of 1.00 and a standard deviation of zero would obtain. As can be seen from the calculations presented in Table XVIII the triacylglycerol values obtained at lipase levels of 3200 and 6000 U/l do not differ significantly by this criterion. A level of 4800 U/l was chosen for the proposed method to allow a margin for safety while operating at a reasonable cost.

The specificity of the lipase used in the proposed method is a major concern, since the detection reaction will respond to fatty acids, whatever the source. The sources of fatty acids of potential concern are phospholipids and cholesterol esters.

The presence of considerable activity against cholesterol esters in lipase from Chromobacterium viscosum (Table X) removed this lipase from consideration for use in the proposed method. No detectable activity against cholesterol esters (Table X) or phospholipids (Table XI) was found using lipase from Rhizopus arrhizus. These findings, in conjunction with the high correlation coefficient between the Calbiochem method and the proposed method, are indicative of the high degree of specificity of the proposed method.

Standardization of serum triacylglycerol methods has long been an area of uncertainty, particularly for the enzymatic methods (Slickers, 1976). Several reasons exist for this problem:

1. The fatty acid content, and thus the molecular weight, of serum triacylglycerols is heterogeneous.
2. The use of synthetic triacylglycerols as standards is subject to substantial error, since the rate at which a lipase hydrolyzes a substrate is proportional to the surface area of the dispersed substrate rather than the concentration of the substrate, per se (Benzonana and Desnuelle, 1965).
3. Although lyophilized serum is the most convenient stable source of serum triacylglycerols for standardization, the

absence of a reference method leads to a chaotic situation when an assay value is assigned to this type of serum. This is illustrated in Table XXVII, which reproduces the insert sheet for a commercial control serum. The range of values listed for the various glycerol detection methods, from 90 to 150 mg/dl, is a sad comment on the "state of the art" in standardization of serum triacylglycerol methods. The method of standardization of the proposed method, when viewed in this perspective, is acceptable.

A substantial difference, averaging 19%, was observed in the assay values obtained on a group of serum specimens before and after lyophilization (Table XX). However, when frozen serum pools were used to standardize the proposed method, no significant difference was obtained between the results using frozen serum pools and the same serum pools before freezing (Table XXI). A recently introduced continuous flow type analyzer (SMAC^{R}, Technicon Corp.) uses frozen, rather than lyophilized, serum for standardization, so this method of standardization is not precluded.

The adaptation of any methodology to a continuous flow type of analyzer requires consideration of a number of parameters unique to this type of instrumentation (Thiers and Oglesby, 1964; Thiers, Cole and Kirsch, 1963). These include the sample to wash ratio, sampling rate, interaction of a serum sample with the preceding and succeeding serum samples, and attainment of a constant percentage of steady state color development. All of these parameters are

Table XXVII

Reproduction of Triacylglycerol Assay Values From an Insert Sheet for a Commercially Available Control Serum

Constituent	Method	Value	Units	Expected Range	Normal	Range
	ACA (Enzymatic)	111	mg/dl	±17	30	200
	BMC (Enzymatic)	103	mg/dl	±12	74	172
	Calbiochem (Enzymatic)	104	mg/dl	±12	45	170
	Coulter Chemistry (Enzymatic)	91	mg/dl	±14	6	177
	Dow (Enzymatic)	60	mg/dl	±9	36	165
	Eskalab (Enzymatic)	102	mg/dl	±15	10	190
Triglycerides	GEMENI (Bucolo-David)	109	mg/dl	±11	0	184
	Harleco (Van Handel-Zilversmit)	95	mg/dl	±14	50	190
	Hycel 17 (Enzymatic)	125	mg/dl	±15	50	155
	KDA (Enzymatic)	64	mg/dl	±10	36	165
	SMAC (Enzymatic)	94	mg/dl	±11	30	170
	TRI-CHOL (Saponification/acetylacetone)	96	mg/dl	±14	50	190
	Worthington (Enzymatic)	81	mg/dl	±12	27	230

[1] Ortho Normal Control Serum lot number 6S022.

somewhat interdependent, since an increase in sampling rate ordinarily causes an increase in the amount of interaction and a decrease in the percentage of steady state color development. On the other hand, an increase in the sample to wash ratio increases interaction, but also increases the percentage of steady state color development obtained. The percent of steady state color development obtained is not critical, as long as the same proportion is obtained throughout the useful range of the method. As can be seen from Table XXIV, this is the case for the proposed method at sampling rates of 50/hr and 100/hr. Interaction values for the proposed method, 1.27% at 50/hr and 2.22% at 100/hr, are well within the range of typical interaction values for AutoAnalyzer II^{R} methods presented by Thiers and Oglesby (1964). The precision of the proposed method compares well with representative precision data for semi-automated methods reported by Whitner, Mann and Witter (1972). The proposed method is linear to at least 550 mg/dl (Figure 11) and, with dilution, useful to at least 1100 mg/dl (Figure 12).

The combination of many of the above considerations, plus several others, led to the choice of the manifold design shown in Figure 10. This manifold offers several advantages to the proposed method. The use of a blank channel minimizes the protein effect and allows compensation for the presence of endogenous fatty acids. The sample and reagent volumes chosen yield a balance among sensitivity, extent of linearity, and minimization of the protein effect. The sampling rate and sample to wash ratio chosen allow

attainment of >95% of steady state color development throughout the useful range of the proposed method (Table XXIV), coupled with adequate levels of interaction and use of a printer, if desired.

Due to the lack of a reference method for serum triacylglycerol assays, comparison with a well accepted method is the only recourse for validation of a new methodology. The method of Bucolo and David (1973), as manufactured by Calbiochem, was chosen. The Calbiochem method is totally enzymatic, and may be corrected for any endogenous glycerol present in the serum specimens. Using the protocol of Westgard and Hunt (1973), the proposed method was compared to the Calbiochem method. Analysis of the data (Table XXV) yields the following conclusions:

1. Proportional error is 0.7% as estimated from the slope for the regression line of 0.993.
2. Constant error is approximately 10 mg/dl as measured by both the intercept for the regression line and the bias. This error is almost identical to the sum of the protein and turbidity effects measured on this group of serum specimens.
3. Random error is 9.63 mg/dl (S_y) and 16.6 (SD_d) and the correlation coefficient is high (0.994). A value of 200 mg/dl by the Calbiochem method would result in a value of 199 ± 19.2 mg/dl (95% confidence limits for S_y) by the proposed method.
4. The scattergram (Figure 12) shows no apparent alinearity in the relationship between the methods over the range of values measured.

5. The large value for t (4.03, $p < 0.001$) is probably due to the combination of a relatively large constant error and a relatively small random error. (See Westgard and Hunt [1973] for a discussion of the interpretation of t values.) In contrast, the comparison of the proposed method to the Harleco method (Mazza and Crowther, 1976) resulted in a t value of 0.99. The constant error in this case is approximately equal to the constant error in the Calbiochem comparison, but the large random error (SD_D = 37.9) introduced by the inclusion of endogenous glycerol in the Harleco values results in a value for t which is not representative of the agreement between methods. The magnitude of the error introduced by the inclusion of the endogenous glycerol in the triacylglycerol value is ordinarily small, averaging less than ten percent of the serum triacylglycerol value (Donabedian, 1974; Tiffany et al., 1974). In the present study, the average endogenous glycerol was 15.7 mg/dl (as triolein), 8.7 percent of the average triacylglycerol value. However, in some samples the endogenous glycerol represented as much as 41.6% of the triacylglycerol value in the present study, and values as high as 94% have been observed during the course of this research. The effect of the glycerol blanks can be seen in Figure 14, where the samples containing an endogenous glycerol level of 20 mg/dl (as triolein) or higher are designated by a "□".

The coincidence between the magnitude of the endogenous glycerol level and the distance from the regression line is readily apparent.

The effect of potentially interfering substances on the performance of the proposed method was not evaluated in depth. However, the addition of sodium salicylate to serum specimens produced no detectable changes in the assay values for triacylglycerols. The sources of the serum specimens, two local hospitals, virtually guaranteed that a wide spectrum of drugs would be represented in the serum specimens. Additionally, a wide range of cholesterol ester values would be expected in the samples. Kucerova (1966) found no displacement of BSP from albumin by bilirubin or bile salts. Finally, the presence of significant quantities of endogenous glycerol, and by analogy, large quantities of endogenous fatty acids, was effectively compensated for by the blank channel. The extremely high correlation coefficient in spite of all of these potential interferences is a good indication of the high specificity of the proposed method.

The performance of the proposed method using fresh reagent has been evaluated above. Another important aspect of the performance is how storage affects the results obtained using the proposed method. For this reason, a stability study was undertaken, as described in the Methods section. The results, shown in Table XXVI, pose a problem. Using the criteria set forth in the Methods section, the reagent is stable at all temperatures tested on every date during the study. This can be seen by comparing the assay

values obtained by the proposed method at each temperature on each testing date. On the other hand, the colorimeter gain had to be changed by large increments in some cases to yield the same assay values. Since adjustment of the colorimeter gain is the suggested method for "standardization" of the AAII, this would not seem to be a serious problem. On the other hand, as the colorimeter gain is raised the signal to noise ratio drops, and the ability to detect small changes in serum triacylglycerol values decreases. Therefore, any criterion for the period of stability of the proposed reagent must take all of these points under consideration.

As previously mentioned, the cost of the enzymatic methods is a barrier to their wider use as a screening tool. The Calbiochem test, when used in the manual mode, following the manufacturer's instructions exactly and correcting for endogenous glycerol, costs $2.78 per sample, exclusive of blanking or standardization. This method has been adapted to the AutoAnalyzer II^{R} (Bucolo, Yabut and Chang 1975), at a cost of approximately 50 cents per test at a sampling rate of 80 per hour. This does not include blanks or standardization. The cost for the proposed method (materials only) ranges from approximately four cents per test at 50 samples per hour to two cents per test at 100 samples per hour on the same basis.

CHAPTER VI

SUMMARY

In summary, the following has been accomplished:

1. Following evaluation of serum albumin from ten species, and evaluation of 40 dyes, bovine serum albumin and sulfobromophthalein were used to develop an aqueous fatty acid detection system, based on the displacement of dye from the albumin by fatty acids.
2. The use of a lipase from Rhizopus arrhizus allowed measurement of serum triacylglycerol content using this principle. The reagent composition is: 4.2 g/l BSA, 200 mg/l BSP, 0.08 µmol/ml oleic acid, 2 g/l sodium azide, 15 ml/l of FC-134 (0.2%) surfactant and 4800 U/l lipase from Rhizopus arrhizus all in 0.1 M sodium pyrophosphate, pH 8.00.
3. The method has been adapted to the AutoAnalyzer II^{R} and operated at sampling rates of 50, 60 and 100 samples per hour.
4. An adequate method of standardization, using frozen serum pools, has been devised.
5. The method is precise (CV < 2.5%), accurate (> 95% recovery, linear to at least 550 mg/dl), and compares well

with the method of Bucolo and David (1973). ($r = 0.994$, $y = 0.993\ x + 10.3$)

6. The percent of steady state color development is the same at a given sampling rate over the useful range of the proposed method.
7. The specificity of the method has been validated.
8. The cost of the proposed method (two to four cents per test for materials, depending on sampling rate) is significantly lower than the cost of methods in present use for the measurement of serum triacylglycerols.

BIBLIOGRAPHY

Albrink, M. J.: Triglycerides, lipoproteins and coronary artery disease. Arch. Intern. Med. 109: 345-359, 1962.

Albrink, M. J. and Mann, E. B.: Serum triglycerides in coronary artery disease. Arch. Intern. Med. 103: 4-8, 1959.

Allain, C. C., Poon, L. S., Chan, C. S. G., Richmond, W. and Fu, P. C.: Enzymatic determination of total serum cholesterol. Clin. Chem. 20: 470-475, 1974.

Altmann, A., Bach, A. and Metars, P.: Détermination enzymatique de la triglycéridémie. Ann. Biol. Clin. 25: 439-452, 1967.

Antonis, A.: Automated techniques in serum lipid analysis. J. Amer. Oil Chem. Soc. 44: 333-340, 1967.

Antonis, A. and Bersohn, J.: The influence of diet on serum triglycerides in South African white and Bantu prisoners. Lancet 1: 3-9, 1961.

Azarnoff, D. L., Esker, M. M. and Brock, F. E.: Micromethod for the determination of serum lipids. J. Lab. Clin. Med. 60: 331-338, 1962.

Baker, K. J. and Bradley, S. E.: Binding of sulfabromophthalein (BSP) sodium by plasma albumin. Its role in hepatic BSP extraction. J. Clin. Invest. 45: 281-287, 1965.

Beng, C. G. and Kim, K. L.: An improved automated method of determination of serum albumin using bromcresol green. Am. J. Clin. Path. 59: 14-21, 1973.

Benzonana, G.: Some properties of an exocellular lipase from Rhizopus arrhizus. Lipids 9: 166-172, 1974.

Benzonana, G. and Desnuelle, P.: Etude cinetique de l'action de la lipase pancreatique sue des triglycerides en emulsion. Eszai d'une enzymologie en melieu heterogene. Biochem. Biophys. Acta 105: 121-136, 1965.

Benzonana, G. and Esposito, S.: On the positional and chain specificities of Candida cylindraceae lipase. Biochim. Biophys. Acta 231: 15-22, 1971.

Blankenhorn, D. H., Rauser, G. and Weiner, T. J.: A method for the estimation of blood glycerides employing florisil. J. Lipid Res. 2: 281-283, 1961.

Buckley, J. A., Deegan, T., Muirhead, R. A. and Williams, M. J.: The application of nephelometry in the estimation of serum and plasma glyceride levels. Clin. Chim. Acta 28: 133-140, 1970.

Bucolo, G. and David, H.: Quantitative determination of serum triglycerides by the use of enzymes. Clin. Chem. 19: 475-482, 1973.

Bucolo, G., Yabut, J. and Chang, T. Y.: Mechanized enzymatic determination of triglycerides in serum. Clin. Chem. 21: 420-424, 1975.

Carlson, L. A. and Wadström, L. B.: Determination of glycerides in blood serum. Clin. Chim. Acta 4: 197-205, 1959.

Carruthers, M. and Young, D. A. B.: Free fatty acid estimation by a semi-automated fluorometric method. Clin. Chim. Acta 49: 341-348, 1973.

Cham, B. E. and Knowles, B. R.: A solvent system for delipidation of plasma or serum without protein precipitation. J. Lipid Res. 17: 176-181, 1976.

Chapman, L. M., Greenberg, D. M. and Schmidt, C. L. A.: Studies on the nature of the combination between certain acid dyes and proteins. J. Biol. Chem. 72: 707-729, 1927.

Chen, R. F.: Removal of fatty acids from serum albumin by charcoal treatment. J. Biol. Chem. 242: 173-181, 1967.

Cogin, G. E. and Davis, B. D.: Competition in the binding of long chain fatty acids and methyl orange to bovine serum albumin. J. Am. Chem. Soc. 73: 3135-3138, 1951.

Cramp, D. G. and Robertson, G.: The fluorometric assay of triglyceride by a semiautomated method. Anal. Biochem. 25: 246-251, 1968.

Deckelbaum, R. J., Tall, A. R. and Small, D. M.: Interaction of cholesterol ester and triglyceride in human plasma very low density lipoprotein. J. Lipid Res. 18: 164-168, 1970.

Donabedian, R. K.: Free glycerol interferences in triglyceride determinations: Warning of a potential problem. Clin. Chem. 20: 632 1974.

Duncombe, W. G.: The colorimetric microdetermination of long chain fatty acids. Biochem. J. 88: 7-10, 1963.

Dunsbach, F.: Die Bestimmung der Triglycerides in Serum mit der Hantzscn-Reaktion. Z. Klin. Chem. 4: 262-264, 1966.

Edwards, L., Falkowski, C. and Chilcote, M. E.: Semiautomated fluorometric measurement of triglycerides. In Standard Methods of Clinical Chemistry, vol. 7, p. 69. Academic Press, New York, 1972.

Eggstein, M. and Kreutz, F. H.: Eine neue Bestimmung der Naturalfette in Blutserum und Gewebe. Klin. Wochenschr. 44: 262-267, 1966.

Finkelstein, A. E., Strawich, E. S. and Sonnino, S.: Characterization and partial purification of a lipase from Pseudomonas aeruginosa. Biochim. Biophys. Acta 206: 380-391, 1970.

Fletcher, M. J.: A colorimetric method for estimating serum triglycerides. Clin. Chim. Acta 22: 393-397, 1968.

Fraenkel-Conrat, H. and Cooper, M.: The use of dyes for the determination of acid and basic groups in proteins. J. Biol. Chem. 154: 239-246, 1944.

Fredrickson, D. S., Levy, R. I. and Lees, R. S.: Fat transport in lipoproteins - an integrated approach to mechanisms and disorders. N. Eng. J. Med. 276: 32-44, 94-103, 148-156, 215-226, 273-281, 1967.

Freeman, N. K.: Simultaneous determination of triglycerides and cholesterol esters in serum by infrared spectrophotometry. J. Lipid Res. 5: 236-241, 1964.

Fukumoto, J., Iwai, M., and Tsujisaka, Y.: Studies on lipase. I. Purification and crystallization of a lipase secreted by Aspergillus niger. J. Gen. Appl. Microbiol. 9: 353-361, 1963.

Fukumoto, J., Iwai, M. and Tsujisaka, Y.: Studies on lipase. IV. Purification and properties of a lipase secreted by Rhizopus delemar. J. Gen. Appl. Microbiol. 10: 257-265, 1964.

Furman, W. B.: Continuous Flow Analysis, Theory and Practice. Marcel Dekker, Inc., New York, 1976.

Galletti, F.: An improved colorimetric micromethod for the determination of serum glycerides. Clin. Chim. Acta 15: 184-186, 1967.

Garland, P. B. and Randle, P. J.: A rapid enzymatic assay for glycerol. Nature 196: 987-988, 1962.

Gomori, G.: A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. 27: 955-960, 1942.

Goodman, D. S.: Preparation of human serum albumin free of long-chain fatty acids. Science 125: 1296-1297, 1957.

Goodman, D. S.: The interaction of human serum albumin with long-chain fatty acid anions. JACS 80: 3892-3898, 1958.

Hassing, G. S.: Partial purification and some properties of a lipase from Corynebacterium acnes. Biochim. Biophys. Acta 242: 381-394, 1971.

Havel, R. J.: Pathogenesis, differentiation, and management of hypertriglyceridemia. Adv. Inter. Med. 15: 117-154, 1969.

Hoover, R., Laurentis, S. F. and Gunitileke, K. G.: Spoilage of coconut oil: Purification and properties of a fungal lipase that attacks coconut oil. J. Amer. Oil Chemistry Soc. 50: 64-67, 1973.

International Marketing Service Survey of Clinical Laboratory Sales, International Marketing Service, Inc., New York, 1977.

Jacobs, S. L. and Henry, R. J.: Studies on the gravimetric determination of serum lipids. Clin. Chim. Acta 7: 270-276, 1962.

Jensen, R. G.: Characteristics of the lipase from the mold Geotrichium candidum: A review. Lipids 9: 149-157, 1974.

Jover, A.: A technique for the determination of serum glycerides. J. Lipid Res. 4: 228-230, 1963.

Kessler, G. and Lederer, H.: Fluorometric measurement of triglycerides. In Automation in Analytical Chemistry, ed. by L. T. Skeggs, Jr., Technicon Symposia, 1965, p. 341. New York, Mediad, 1966.

Klotz, I. M.: Spectrophotometric investigations of the interactions of proteins with organic anions. J. Amer. Chem. Soc. 68: 2299-2304, 1946.

Klotz, I. M., Triwush, H. and Walker, F. M.: Binding of organic ions by proteins - competition phenomena and devaluation effects. J. Am. Chem. Soc. 70: 2935-2941, 1948.

Klotz, I. M. and Walker, F. M.: The binding of organic ions by proteins: Change and pH effects. J. Am. Chem. Soc. 69: 1609-1612, 1947.

Kucerová, L., Hoenig, V., Jirsa, M. and Fabian, E.: Die Bindung in vitro von Bromsulfphtalein, Fettsäuron, Salzon der Gallensäuren und von Bilirubin on Albumin. Acta Hepato-Splenol 13: 281-288, 1966.

Laurell, S.: A method for routine determination of plasma triglycerides. Scand. J. Clin. Lab. Invest. 18: 668-672, 1966.

Lofland, H. B. Jr.: A semiautomated procedure for the determination of triglycerides in serum. Ann. Biochem. 9: 393-400, 1964.

Malangeau, P. and Pays, M.: Dosage des glycerides sanguins. Ann. Biol. Clin. 25: 845-851, 1967.

Mattson, F. H. and Volpenheim, R. A.: Hydrolysis of primary and secondary esters of glycerol by pancreatic juice. J. Lipid Res. 9: 79-84, 1968.

Mazza, J. C. and Crowther, F.: A direct enzymatic method for the determination of serum triglycerides using glycerol dehydrogenase. Clin. Chem. 22: 1195, 1976.

Mendelsohn, D. and Antonis, A.: A fluorimetric micro glycerol method and its application to the determination of serum triglycerides. J. Lipid Res. 2: 45-50, 1961.

Michaels, G.: A method for the determination of plasma glycerides and free fatty acids. Metabolism 11: 833-839, 1962.

Nillson-Ehle, P., Belfrage, P. and Bergström, G.: Purified human lipoprotein lipase: Positional specificity. Biochim. Biophys. Acta 248: 114-120, 1971.

Randrup, A.: A specific and reasonably accurate method for routine determination of plasma triglyceride. Scand. J. Clin. Lab. Invest. 12: 1-9, 1960.

Rawlins, L. M. C. and Schmidt, C. L. A.: The mode of combination between certain dyes and gelatin granules. J. Biol. Chem. 88: 271-284, 1930.

Reynolds, J., Herbert, S. and Steinhardt, J.: The binding of some long-chain fatty acid anions and alcohols by bovine serum albumin. Biochemistry 7: 1357-1361, 1968.

Rice, E. W.: Triglycerides (neutral fat) in serum. In Standard Methods of Clinical Chemistry, vol. 6, p. 215. Academic Press, New York, 1970.

Royer, M. E. and Ko, H.: A simplified semi-automated assay for plasma triglycerides. Anal. Biochem. 29: 405-416, 1969.

Rutstein, D. D., Ingenito, E. F. and Reynolds, W. E.: The determination of albumin in human blood plasma and serum. A method based on the interaction of albumin with an anionic dye 2-(4'-hydroxy-benzoneazo) benzoic acid. J. Clin. Invest. 33: 211-221, 1953.

Ryan, W. G. and Rasho, O. M.: An improved extraction procedure for the determination of triglycerides and cholesterol in plasma or serum. Clin. Chem. 13: 769-772, 1967.

Sanabar, S. S.: Hyperlipidermia and Hyperlipoproteinemia, p. 2. Little, Brown & Co., Boston, 1969.

Sardesia, V. R. and Manning, J. A.: The determination of triglycerides in plasma and tissues. Clin. Chem. 14: 156-161, 1968.

Scatchard, G. and Black, E. S.: The effect of salts on the isoionic and isoelectric points of proteins. J. Phys. Colloid Chem. 53: 88-99, 1949.

Schmidt, F. H. and von Dahl, K.: Method for enzymic neutral fat determination in biological material. Z. Klin. Chem. Klin. Biochem. 6: 156-159, 1968.

Shastry, B. S. and Raghavendra Rao, M. R.: Studies on rice bran lipase. Indian J. Biochem. Biophys. 8: 327-332, 1971.

Slickers, K. A.: Enzyme linked assays for serum cholesterol and triglyceride determinations. Lab World 27: 8-15, 1976.

Smith, E. B.: The relationship between plasma and tissue lipids in human atherosclerosis. Adv. Lipid Res. 13: 1-49, 1975.

Soloni, F. G.: Simplified manual micromethod for determination of serum triglycerides. Clin. Chem. 17: 529-534, 1971.

Spector, A. A.: Fatty acid binding to plasma albumin. J. Lipid Res. 16: 165-179, 1975.

Spinella, C. G. and Mager, M.: Modified enzymatic procedure for the routine determination of glycerol and triglycerides. J. Lipid Res. 7: 167-169, 1966.

Stavrapolous, W. S. and Crouch, R. D.: A new colorimetric procedure for the determination of serum triglycerides. Clin. Chem. 8: 857, 1974.

Steinhardt, J. and Reynolds, J. A.: Multiple Equilibria in Proteins. Academic Press, New York, 1969.

Sugiura, M. and Isobe, M.: Purification of a low molecular weight lipase and its enzymatic properties. Biochim. Biophys. Acta 341: 195-200, 1974.

Thiers, R. E. and Oglesbly, K. M.: The precision, accuracy and inherent errors of automatic continuous flow methods. Clin. Chem. 10: 246-257, 1964.

Thiers, R. E., Cole, R. R. and Kirsch, W. J.: Kinetic parameters of continuous flow analysis. Clin. Chem. 13: 451-467, 1967.

Tietz, N., ed.: Fundamentals of Clinical Chemistry, pp. 518-519. W. B. Saunders Company, Philadelphia, 1976.

Tiffany, T. O., Morton, J. M., Hall, E. M. and Garret, A. S., Jr.: Clinical evaluation of kinetic fixed time and integral analysis of serum triglycerides. Clin. Chem. 20: 476-481, 1974.

Timms, A. R., Kelly, L. A., Spirito, J. A. and Engstrom, R. G.: Modification of Lofland's colorimetric semiautomated serum triglyceride determination, assessed by an enzymatic glycerol determination. J. Lipid Res. 9: 675-680, 1968.

Trout, D. L., Estes, E. H. Jr. and Friedberg, S. J.: Titration of free fatty acids of plasma: A study of current methods and a new modification. J. Lipid Res. 1: 199-202, 1960.

Vadehra, D. V.: Staphylococcal lipases. Lipids 9: 158-165, 1974.

Van Handel, E. and Zilversmit, D. B.: Micromethod for the direct determination of serum triglycerides. J. Lab. Clin. Med. 50: 152-157, 1957.

Vanzetti, G. and Denegri, E.: Determination of neutral plasma glycerides. Giom. Biochim. 13: 405-414, 1964.

Wahlfeld, A., Klose, S. and Munz, E.: Fully enzymatic triglyceride determination (UV) in serum - a single channel method for the AA II and SMA Auto-Analyzer. Clin. Chem. 21: 943, 1975.

Westgard, J. O. and Hunt, M. R.: Use and interpretation of common statistical tests in method-comparison studies. Clin. Chem. 19: 49-57, 1973.

Westphal, U., Stets, J. F. and Priest, S. G.: Influence of fatty acids and related anions on the azarubin-binding capacity of serum albumin. Arch. Biochem. Biophys. 43: 463-471, 1953.

Whitner, V. S. Mann, A. N. and Witter, R. F.: Determination of serum triglycerides. In Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism, ed. by G. J. Nelson. Wiley Interscience, New York, 1972.

Yert, A. L., Mather, A., Whitner, V. S. and Witter, R. F.: Determination of serum triglycerides. In Blood Lipids and Lipoproteins: Quantitation, Composition and Metabolism, ed. by G. J. Nelson. Wiley Interscience, New York, 1972.

GRADUATE SCHOOL
UNIVERSITY OF ALABAMA IN BIRMINGHAM
DISSERTATION APPROVAL FORM

Name of Candidate Louis J. Dunka, Jr.

Major Subject Biochemistry

Title of Dissertation An Automated Method for the Determination of Serum Triacylglycerols

Dissertation Committee:

Leo M. Hoel, Chairman
John M. McKibbin
Gerald L. Carlson
Edwin Bradley, Jr.
John L. Segal

Director of Graduate Program John M. McKibbin

Dean, UAB Graduate School Kenneth J. Roozen

Date