DETERMINATION OF VITAMIN E ISOMERS IN ERYTHROCYTES AND PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A COMPARISION OF NORMAL AND SICKLE CELL PATIENTS' LEVELS

by

DARLENE A. VASBINDER

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PRESENTED BY DARLENE A. VASBINDER

ACCEPTED BY THE DEPARTMENT OF CHEMISTRY

Leonard S. Spiegel 8/15/87

Major Professor Date

8/19/87

Date

Daryl W. Maney 8/19/87

Date

Dean, Graduate School Date

ABSTRACT

DETERMINATION OF VITAMIN E ISOMERS IN ERYTHROCYTES AND PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: A COMPARISION OF NORMAL AND SICKLE CELL PATIETNTS' LEVELS

Darlene A. Vasbinder

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This thesis describes the development and evaluation of a High Performance Liquid Chromatography procedure for the determination of the isomers of vitamin E in red blood cells and plasma of humans. Vitamin E is a fat-soluble vitamin, known to have a protective role as an antioxidant. Various procedures for vitamin E determination in the literature were studied and evaluated in' this research. The method developed was a combination of several HPLC methods currently in use, and was evaluated for linearity, reproducibility (both within run and between-run), and precision. In addition, recovery studies were also performed for alpha- and ganuna-tocopherol.

The isomeric forms of vitamin E were quantitated in both the red blood cells and plasma as alpha, beta plus gamma, and delta. In addition to this quantitation, a comparision was made between the levels in normal patients and the levels in patients homozygous for Sickle Cell Disease. Various statistical parameters were used for this comprehensive comparision. The results of the study show that significant differences do indeed exist between normal individuals' and Sickle Cell Disease patients' red blood cell gamma-tocopherol and both alpha-and total tocopherol in the plasma. Suggestions for further work are also discussed.

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LIST OF SYMBOLS

SYMBOL	DEFINITION
α	Alpha
β	Beta
γ	Gamma
δ	Delta
k'	Capacity Factor
	Theoretical plates
HPLC	High Performance Liquid Chromatography
Hgb	Hemoglobin
ISC's	Irreversibly Sickled Erythrocytes
v_r	Retention volume
v_o	Void volume
t_r	Retention time of retained sample
t_o	Retention time of non-retained sample
HETP	Height Equivalent to a Theoretical Plate
	Resolutuion
	Widths of Peaks at Their Bases
nm	Nanometers (10^{-9} m)
EDTA	Ethylenediaminetetra-acetic acid

SYMBOL	DEFINITION
OD	Outer Diameter
ID	Inner Diameter
RBC	Red Blood Cell
PUFA	Polyunsaturated fatty acids

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CHAPTER I

INTRODUCTION

A. Definition and Structure of the Four Naturally Occurring Tocopherols

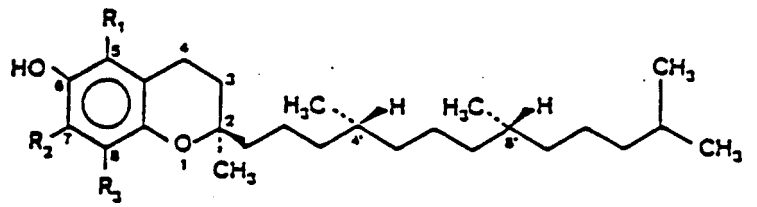
Vitamin E was recognized as an essential nutrient as a result of the studies of Evans and Bishop (1). Structurally, the tocopherols consist of a heterocyclic chromanol ring with a 16-carbon aliphatic side chain. The naturally occurring tocopherols alpha, beta, gamma, and delta tocopherol, differ in the number and position of the methyl groups on the chrananol ring, as shown in Figure 1 on the following page. This structure is a disguised version of a 1,4-dihydroquinone in which one of the hydroxyl groups has been converted to an ether. It also resembles Coenzyme Q and may be involved with this enzyme in mitochondrial oxidation.

B. Occurrence

4-Tocopherol is the predominant isomer in plasma, tissues, and red blood calls. In human tissues, approximately 88% of the total vitamin E is d-α tocopherol, 2% d-β-tocopherol, and 10% d-γ-tocopherol. ζ-tocopherol is present in extremely small quantities (2).

Since humans are unable to synthesize this vitamin, they must rely on external sources such as vegetable oils, nuts and seeds, green leafY vegetables, egg yolks, legumes, and margarine. The adult daily requirement is 8 mg for women and 10 mg for men, as given by the Food and Nutrition Board, but it may be as little as 5 mg. or as much as 30 mg. (1).

Figure 1. STRUCTURE OF TOCOPHEROL



Compound	R_1	R_2
4-Tocopherol	CH_3	CH_3
β-Tocopherol	CH_3	H
γ-Tocopherol	H	CH_3
δ-Tocopherol	H	H

C. Metabolism

Tocopherol is one of the fat-soluble vitamins and like other vitamins of the same classification, its absorption, transport, storage and metabolism are only partially understood.

Absorption of this vitamin may be related to the absorption of intestinal fat. As much as 40% of tocopherol that is ingested is absorbed and this percentage is affected by the amount of unsaturated dietary fat (3) . As tocopherol is absorbed, it is associated with circulating chylomicrons and very low density lipoproteins (VLDL) and eventually with other lipoproteins. Absorbed vitamin E is found chiefly in adrenal tissue (4) and next in adipose tissue, although increased dietary intake increases the concentration found in all tissues including the plasma, erythrocytes and platelets.

D. Function

The exact role of vitamin E in biological processes remains controversial. The description best agreed upon by biochemists establishes this vitamin as an antioxidant, protecting-unsaturated lipids from peroxidation. By combining with free radicals to form a relatively stable hydroquinone, vitamin E prevents oxidative damage to lipid membranes. This suggested role for the tocopherols provides an explanation for the hemolysis that occurs in the anemia associated with vitamin E deficiency and for the sensitivity of red blood cells to substances such as peroxide and iron (Fe^{+3}) that are capable of catalyzing or inducing lipid peroxidation. It has also been suggested that vitamin E is a structural component of biological membranes (1).

Vitamin E has been shown to inhibit platelet aggregration (5,6). In addition, it has been shown to affect specific functions of the mitochondrion, suggesting a role in the electron transport chain. Other proposed roles include the slowing of the aging process, enhanced athletic ability, increased sexual endurance, prevention of heart conditions, and a role in drug metabolism (3). In summary, the role of this vitamin in protecting the erythrocyte biological membranes from oxidant stress is presently the major role of the vitamin in human physiology.

. Clinical Deficiency

Vitamin E deficiency is rare in man, probably due in part to the wide distribution of the vitamin in foodstuffs. Those diets high in polyunsaturated fatty acids (PUFA) increase the body's need for vitamin E. However, even with this, the vitamin is exceptionally well-stored. One study showed adult men experienced no symptoms of anemia after three years on a deficient regimen (7).

In premature infants, vitamin E deficiency has been related to hemolytic anemia. As a result, such infants are giuen supplements of vitamin E. This deficiency associated anemia further relates the vitamin to a role in the maintenance of membrane integrity.

All known cases in humans of vitamin E deficiency are apparently due to defective lipid absorption or transport rather than due to reduced dietary intake. At the present time, assessment of vitamin E status is indicated in the premature infant and in those adults receiving synthetic diets. The classic signs of vitamin E deficiency include creatinuria, muscle weakness, ceroid deposition, increased serum creatine phosphokinase and subnormal levels of tocopherol in the serum (3).

F. Toxicity

Toxic effects suggested for humans include inhibition of prostoglandin synthesis, decreased platelet aggregation, potentiation of coagulopathy (from vitamin K deficiency), weakness and fatigue with creatinuria, inhibition of fibrinolysis and impaired wound healing (3).

G. Overview of Sickle Cell Anemia

Sickle cell anemia was first described by a Chicago physician, James Herrick, in 1910 (8). The patient whose case history he described was a twenty-year-old black male from the West Indies who presented with most of the classic clinical and hematologic features of the disease. Since this first documentation of the disease, much additional information has been gained . The intent of this paper is to further contribute to

that body of information currently available regarding the vitamin E levels in the erythrocytes and plasma of these patients in comparision to normal patients. This is part of an effort to further elucidate a possible mode of treatment for the victims of this disease.

Shortly after the publication of Herrick's case report, other cases of this disease were found. Indeed, it was found that this disease was not a rare occurrence. The incidence of sickle cell anemia among American blacks is about four per thousand (9). In the past, sickle cell anemia was associated with a high fatality rate, often before the age of thirty. Death usually resulted from infection, renal failure, cardiac failure, thrombosis (bone marrow and fat emboli), shock, complications in the central nervous system, tuberculosis, or from sarcoidosis (10).

The abnormally shaped erythrocytes become trapped in the small blood vessels, impairing circulation and resulting in damage to the organs, especially the kidneys. The resultant hemolysis of the cells causes them to have a much shorter lifespan than normal red blood cells. Such hemolysis results in anemia that may vary in severity, punctuated by crises in which the ratio of 'sickled' erythrocytes to normal erythrocytes is extremely high. It is during such times that the patient may go into shock as a consequence of the anemia. Sickle cell patients' mean hemoglobin value is 7.5 g/dL with a range of 5.5-9.5 g/dL (1). This is approximately one half of the normal values (11). (See Table 1.)

It must be emphasized that "normal" is an arbitrary term and that in any study of "normal" subjects, a few who may not be truly in perfect health are likely to be included.

Table 1. Adult Normal Values

	Red cell count (million/mm^3)	Hemoglobin (Mean)	S.D.
Adult White Male	4.2–5.4	14.4	1.21
Adult White Female	3.6–5.0	13.1	1.25
Adult Black Male	3.8–5.2	14.0	1.25
Adult Black Female	3.1–4.5	12.4	1.43

Sickle cell anemia is genetically transmitted and those patients that are homozygous for the abnormal gene exhibit the disease. The abnormal gene is located on one of the autosomal chromosomes. Those offspring that receive only one abnormal gene and a normal allele are heterozygous and have what is known as sickle cell trait. These individuals generally are asymptomatic. Sickle cell trait and anemia have been found almost entirely in the black race. The incidence of the trait varies in different parts of the world, but is about 9% in American blacks. Sickle cell anemia has a frequency of about one fortieth that of the trait (12). Approximately 1% of the red cells in heterozygotes'

venous circulation are sickled, in contrast to about 50% in a homozygote (9).

In hemoglobin S, the amino acid, Glutamic Acid, in the sixth position in the Beta chain is replaced by Valine. This change occurs on the outer surface of the molecule, changing its charge and electrophoretic mobility (10). The exact location of this alteration is the amino-terminal tryptic peptide of the beta chain.. Ingram's determination of the sequence of this peptide is shown below (9).

Figure 2. Hemoglobin Sequence in Normal and Sickle Hemoglobin

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Hemoglobin A: Val-His-Leu-Thr-Pro-Glu-Glu-Lys

Hemoglobin S: Val-His-Leu-Thr-Pro-Val-Glu-Lys

The deoxyhemoglobin S forms fibrous precipitates that deform the red cells, resulting in their sickled shape. Two types of fibers have been seen by electron microscopy: one fiber with a diameter of 17 nm and, more frequently, one with a diameter of 21.5 nm. The more prevalent precipitate is a fourteen stranded helix built from ten external and four internal hemoglobin S molecules. In this helix, each hemoglobin S molecule makes contact with at least eight others. This fiber is stabilized by multiple interactions: one involving valine 6 of the beta chain tips the thermodynamic balance of the deoxygenated state toward fiber formation. The formation of deoxyhemoglobin S fibers in a

red cell in capillary circulation takes about a second (9). The process of sickling is reversible when the hemoglobin is reoxygenated. However, upon repeated cycles of sickling and unsickling, the red cell membrane becomes damaged and no longer permits the cell to return to a biconcave shape. These cells are then termed irreversibly deformed cells and are called irreversibly sickled cells (ISCs) (13).

Cellular breakdown has been related to peroxidative reactions (14). Circulating red blood cells are particularly susceptible to peroxidative damage because the membranes of these cells are rich in polyunsaturated fatty acids. These cells are continually exposed to high oxygen tensions, and the cells contain hemoglobin, one of the most powerful catalysts for the initiation of peroxidative reactions. Normal red cells are protected in vivo by efficient antioxidant mechanisms. These include the following defense mechanisms: superoxide dismutase, glutathione peroxidase, catalase, and vitamin E. A failure of any of these may render the erythrocyte more susceptible to peroxidative damage and eventually lead to its demise.

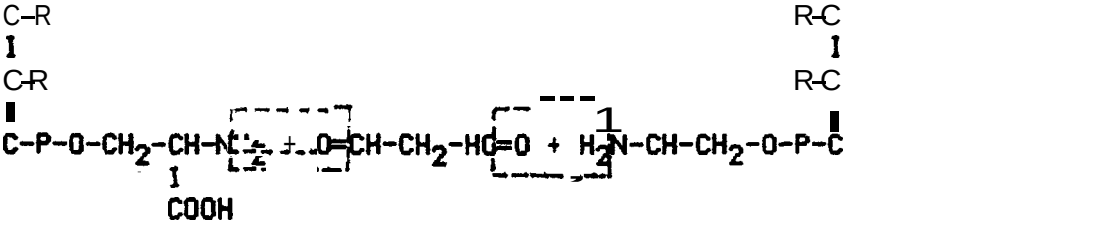
Chiu et al (14) observed that a major distortion of the red cell shape occurred upon sickling and this indicated a loss of structural integrity that was perhaps due to an increased susceptibility of sickled erythrocytes to peroxidation. Their study confirmed this and demonstrated that membrane phospholipid reorganization occurs during this sickling process with an increased quantity of phosphatidylethanolamine and phosphatidylserine exposed to the outer lipid bilayer. They felt

that it was conceivable for such abnormal membrane phospholipid organization to render sickle cells more susceptible to lipid peroxidative damage.

However, this same study revealed that increased susceptibility to peroxidation was not entirely due to abnormal membrane lipid asymmetry. They found that even under oxygenated conditions, sickled erythrocytes are still more susceptible to lipid peroxidation than are normal red blood cells. Chiu et al (14) felt that this suggested an abnormality in the antioxidant system and the additional abnormality was identified as low serum levels of vitamin E.

Shohet and Jain (15) have further demonstrated that sickle hemoglobin when autooxidized, produces uarious oxidation products that affect membrane fatty acids and produce malonyldialdehyde (MDA), a final product of membrane lipid peroxidation. The cross - linking of phosphatidylserine and phosphatidylethanolamine with malonyldialdehyde is shown on the following page.

Figure 3. Cross-linked Lipid Adduct



Since vitamin E has a role in moderating peroxidatiue reactions in biological systems, it was expected that more of the above adduct would be found in conditions with decreased red blood cell vitamin E. At least with regard to sickle cell anemia and iron deficiency anemia, this group found this to be the case (15). Thus, this cross-1inked lipid may play some role in the physiologic abnormalities of the sickled erythrocytes seen in sickle cell anemia. Once again, reduced vitamin E levels were associated with this Beta hemoglobinapathy.

Mino et al (16) showed that the hemolysis occurring in red blood cells may be more directly related to the tocopherol level in red blood cello than in plasma. They also demonstrated that the transportation of tocopherol from plasma to red blood cell membranes increased when the tocopherol concentration in plasma lipids increases. The work of this group further confirms an association between vitamin E levels and red blood cell integrity, but proceeded to suggest that the red blood cell level of the

vitamin was more important than the plasma level in determining this integrity.

Natta et al(17) found that the levels of tocopherol in plasma from sickle cell anemia patients were law. This correlates with other research previously mentioned. However, this group proceeded one step further and observed changes in the number of circulating ISCs in sickle cell patients after daily treatment with 450 I.U. of vitamin E . The results of their work showed that this administration of vitamin E reduced the number of circulating ISCs from mean values of 25% to 11% with no change in hemoglobin levels (18). Thus, the study by Natta et al. suggested the parenteral administration of vitamin E as a means to restore the sickle cell patients' vitamin E level to normal and to subsequently reduce the level of irreversibly sickled red cells. Since membrane changes result in changes in the shape of the erythrocytes, and since all of the tocopherol in the erythrocyte is located in the membrane, this study further confirms that vitamin E levels are intimately related to membrane integrity and that tocopherol apparently stabilizes the erythrocyte membrane against oxidative stress.

The determination of tocopherol in the plasma and erythrocytes of normal individuals and sickle cell disease patients has been performed by several groups of researchers (19). The determination of α-tocopherol in tissue, plasma, and erythrocytes can be performed by a variety of methods.

H. Statement of the Problem

The purpose of this research is to improve the existing fluorescent methods for the detection of alpha, beta and gamma, and delta-tocopherols in both the plasma and red cells and subsequently, determine the level of these components in normal individuals and subjects known to have Sickle Cell disease. The basis for this determination is from work previously done by Taylor, Lamden, and Tappel (20). The extraction method developed by this group was used. The subsequent hexane extract was injected into an IBM High Performance Liquid Chromatograph and the isomeric components separated via a Waters' radial compression system using a reverse-phase C_{18} column recommended by the work of Janine Denis-Cook (21). This system allows the separation of alpha, beta and gamma, and delta tocopherol. Detection was then performed with fluorescent monitoring at 330 nm and the resulting analysis of peak areas allowed quantitation of the respective isomers. The retention times of the various peaks were determined by comparision with known standards for each of the tocopherol components. Quantitation of the peak areas then permitted determination of the amounts of each component in the patients red cello and plasma.

CHAPTER II

REVIEW OF THE LITERATURE

Vitamin E is present in both the plasma and cellular fraction of whole blood. Within the erythrocyte, the majority of this vitamin is found to be associated with the cellular membrane. Various laboratory techniques have been used to assess the level of tocopherol in its assorted forms. In this presentation, an overview of these methodologies will be discussed, with an emphasis on those methods that utilize High Performance Liquid Chromatography with accompanying fluorometric detection.

A. Extraction Methods for Sample Preparation

The determination of tocopherol in biological materials? including red blood cells, initially involves the extraction of lipid materials from the sample. Direct extraction of the total lipid is generally accomplished by selection of the proper solvent(s). Commonly used solvents for this stage of preparation include ethanol, hexane, diethyl ether, chloroform/methanol and acetone, or a combination of the preceding solvents (22).

The next step in sample preparation is saponification and is necessary to convert any tocopherol esters to the free form and to remove fats (especially triglycerides) and other interfering substances before analysis. Basically, all methods require mixing the sample with an appropriate solvent (e.g., ethanol) and potassium hydroxide solution for variable times at specific temperatures. At this point, most procedures introduce the use of an antioxidant, either pyrogallol or ascorbic acid, in order to eliminate the oxidation of tocopherol that occurs under alkaline conditions. Heat extraction and saponification ensure membrane degeneration and release of the free tocopherol so that maximum determination of tocopherol is achieved.

The final preparatory step is reextraction of the lipids with a nonpolar solvent such as hexane. This is performed after saponification and the organic layer thus isolated is usually evaporated to dryness, with the resulting residue then reconstituted with an organic solvent.

B. High-Performance Liquid Chromatographic Methods (HPLC)

High Performance Liquid Chromatography is a technique used to separate the components of a chemical mixture. These solutes are first dissolved in a liquid solvent and then forced through a particular chromatographic column under high pressure (23). The separation of the chemical mixture occurs as a result of this

column. The chromatographic process is thus a moving liquid phase percolating over the stationary bed of the column. The interaction of the solute with mobile and stationary phases can be manipulated through different choices of solvents and materials. HPLC creates a high degree of versatility and allows the easy separation of a wide variety of chemical mixtures, generally in a shorter time period than the other chromatographic methods.

Several HPLC techniques are available, including partition, both 'normal' and 'reverse' phase, size exclusion HPLC, ion-exchange HPLC, and adsorption HPLC. The partition mode is most widely used for routine HPLC. In this method, solute components distribute themselves between the mobile and stationary phases. As a result of the differences in solubility, the solutes elute from the column separately as long as the correct mobile phase is used.

'Normal' partitioning was the first separation technique studied (23). The required conditions for normal separations are a polar stationary phase for separation of nonpolar compounds, and a nonpolar mobile phase. The bonded "reverse" phase partitioning mirrors the normal phase conditions, that is, the stationary phase is nonpolar and the mobile phase is polar.

For efficient liquid chromatography separations, a column must have the following characteristics: 1). the capacity to retain samples, 2). the ability to separate sample components, and finally, 3). must operate efficiently. The following

expression describes the capacity factor (or k') of a column - this is a measure of sample retention by the column:

$$k' = \frac{v_r - v_o}{v_o} = \frac{t_r - t_o}{t_o} \qquad (1)$$

The hold up time or void volume (mobile phase interstitial volume) , (v_o), is the volume of the column unoccupied by the packing and is equal to the total volume of the solvent eluting from the column between the time of injection and the appearance of an unadsorbed species. t_r is the retention time and is the time from injection to that time at which the maximum of the unretained solute peak appears on the recorder. v_r is retention volume (24).

Efficiency is used to describe the potential separation capabilities of the chromatographic system. The results are expressed in terms of the number of theoretical plates, (N), which can be thought of as a certain number of separation stages. The more stages, the higher the efficiency. The following expression describes this:

$$N = 16\left(\frac{t_r}{w}\right)^2 \qquad (2)$$

Where, N = the number of theoretical plates

t_r = retention time

w = baseline width

For good resolution, narrow baseline widths are desired. In order to compare the efficiencies of different length columns, the height equivalent to a theoretical plate, (HETP), is used instead of N. This is easily calculated by dividing (N) into the column length (L). The result is expressed in units of length and for HPLC, the typical HETP value lies between 0.3 and 0.1 mm.

$$HETP = L/N \qquad (3)$$

The ease of separating two different components on a column is given by the selectivity term, α. It is calculated from the ratio of k' values and is always expressed as a number greater than one.

$$\alpha = \frac{k'_2}{k'_1} \qquad (4)$$

Where k'_1 and k'_2 are the capacity factors of each component. Conceptually, α represents the spacing between peak maxima. A largo α (wide spacing) indicates the components separate with more efficiency. As α approaches unity (1), tho peaks fuse and there is no separation.

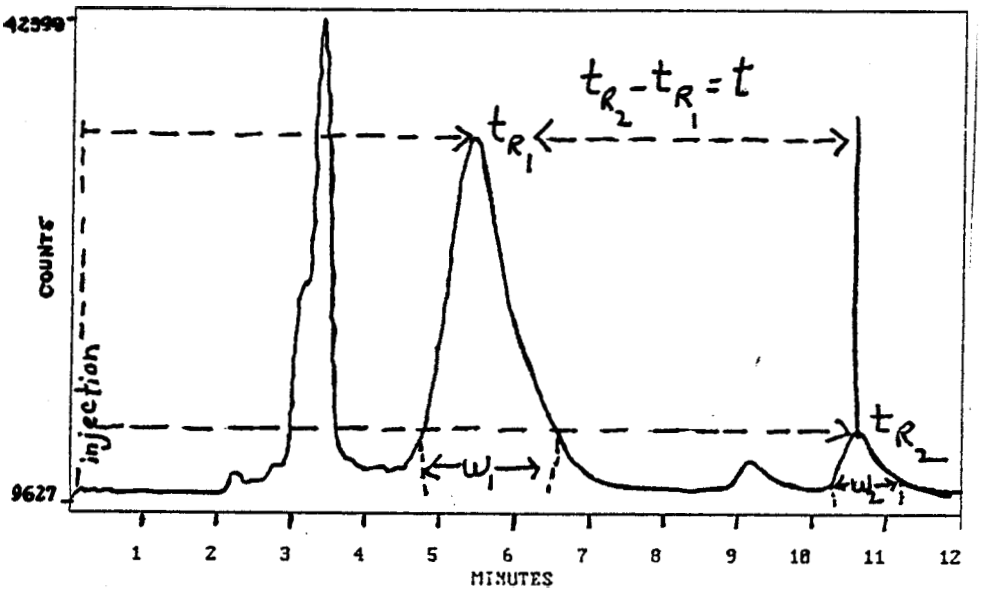
The resolution, or separation, between ouer1apping bands is estimated by the following expression (24) :

$$R = \frac{2\Delta t}{(w_1 + w_2)} \qquad (5)$$

whore A t is the difference between retention times and w is the widths in units of time of the peaks 1 and 2 at their bases.

An illustration showing the relationship of the various chromatographic terms thus described is shown on the following page:

Figure 4. Typical Chromatogram



HPLC can be used to separate components by choosing an eluent system that is either isocratic or gradient. Isocratic elution techniques use constant solvent composition to move the injected sample through the column. Gradient elution uses varying solvent composition for a given length of time in order to separate components of the chemical mixture. Generally, gradient elution utilizes increasing solvent or ionic strength from beginning to the end of the chromatographic run.

Nelis (2) reports that as early as 1975, Abe and coworkers reported an adsorption chromatography method with fluorescent detection to simultaneously determine alpha-, beta-, and gamma-tocopherol in serum and red blood cells. Nelis also described that the first papers specifically dealing with useful alpha tocopherol assays were published by De Leenheer et al and Nilsson et al. The former authors introduced the chromatography of alpha-tocopherol on reversed phase rather than silica columns, and monitored the eluate with ultraviolet detection at 292 nm. Whereas, Nilsson et al. utilized a straight phase system, based on a pellicular Corasil silica column. Their method had numerous drawbacks, among which were the inability to separate gamma from beta tocopherol coupled with poor efficiency and sensitivity.

Later studies abandoned the use of silica in favor of reverse-phase supports. If tocopherols other than alpha are to be determined simultaneously, fluorescent detection is often indicated in order to detect the minor beta species. Fluorescent

detection was also found to be superior to ultraviolet detection in terms of sensitivity and selectivity.

In exceptional cares where still higher sensitivity is required, electrochemical detection may be tried. A reversed-phase liquid chromatographic determination of alpha-tocopherol in plasma using amperometric detection has been described by Ikenoya et a1 (25). Sensitivity of this assay is in the picogram range, and no interferences were noted.

Various assay methods have been investigated for the analysis of tocopherol in both plasma and red blood cells, Desai (19) reviewed these methods including colorimetry, spectrophotometry, spectrofluorometry, column chromatography, paper chromatography, thin layer and gas-liquid chromatography, and, finally, electrochemical methods. Sane literature that dercribes the isolation and quantitation of tocopherols is discussed below, with special emphasis being placed on High Performance Liquid Chromatographic methods.

Before discussing methods, one should be made aware of three major pr-oblems associatedwith vitamin E assays in -- biological materials. The first is related to the instability of tocopherol. Oxidative destruction of the molecule occurs with heat, light, metal ions, and alkaline pH conditions. Various steps can be taken to reduce these risks considerably. A second major interference is caused by the presence of lipids in excess, particulary cholesterol. The final challenge occurs when one tries to separate the positional isomers, beta and gamma.

Therfore, each method represents an attempt to overcome these problems and to analyze tocopherol in an accurate, sensitive and efficient manner.

Hatam and Kayden (26) separated tocopherols in erythrocytes using a microparticlulate reverse phase partition column, u Bondapak C_{18}. They described a high performance liquid chromatographic method that utilized fluormetric detection with an excitation wavelength of 205 m rather than the usual 295 nm.This change achieved a tewnty-fold gain in sensitivity for the assay. This method utilized as little as 50 uL of plasma or 200 uL of erythrocytes for the separation and quantitation of the isomers.

Taylor, Lamden, and Tappel (20) revised Hatam and Kayden's method (26) by altering the excitation and emission wavelengths to 290 m and 330 nm, respectively, and utilized a neutral alumina column onto which the hexane extract was injected. The principle advantage of this method was the elimination of cumbersome and quantitatively inefficient chromatographic procedures in order to separate tocopherol from interfering substances. They felt that this methodology overcame problems generally associated with fluorometric analysis of alpha tocopherol and thus, expanded their technique to the determination of alpha-tocopherol in homogenates, subcellular fractions, and red blood cells.

A method for detection and quantitation of plasma and erythrocyte alpha- and gamma-tocopherol was developed jointly by Lehmann and Martin (27). This method treated 50 uL of red cells

or plasma and required a chloroform extraction followed by evaporation and dissolving the residue in methanol. Because their procedure did not utilize alkaline saponification as other methods listed previously, they selected tocol as an internal standard for monitoring sample loss due to manipulation.

Lehmann and Martin (27) felt that neither saponification nor hexane extraction was necessary, and the resulting method revealed good reproducibility, precision and analytical recovery. This assay also provided advantages over other methods in terms of sensitivity and reproducibility.

Chiu and Lubin (28) modified the method of Taylor's group in such a way that the saponification mixture was altered to require a smaller sample size. This group also chose to use heparinized blood samples as opposed to EDTA samples.

Cook (21) developed a reverse phase High Performance Liquid Chromatography method to simultaneously determine serum alpha-, beta- and gamma-, and delta-tocopherol along with retinol. Her method utilized ultraviolet detection at 280 nm, and she felt that it compared well with Lehmann and Martin's method, but less well with the non-specific fluorometric and colorimetric methods.

The above methods represent a summary of those currently available for HPLC analysis and fluorometric detection of plasma and red cell tocopherol. This author chose to use a combination of the methods used by Cook, Taylor, and Chiu and Lubin for the determination of tocopherol isomers in plasma and red cells. The method was evaluated for precision, accuracy, reproducibility, and

linearity of the isomers of vitamin E. In addition, normal levels were determined for both plasma and cells, and these were then compared to the levels found in Sickle Cell Disease patients.

CHAPTER III

MATERIAL AND APPARATUS

A.) Solvents and Reagents

All solvents and chemicals used were of the highest purity available.

Doubly deionized ultrafiltered water was used for the preparation of the mobile phase and in sample preparation.

Solvents and reagents were prepared or used as described below:

HPLC grade methanol and hexane were further filtered through nylon filters of 0.45 µm pore size. These reagents were purchased from Fisher Scientific Company, Fair Lawn, New Jersey.

Potassium Hydroxide Pellets AR, (Mallinckrodt, Inc. Paris, Kentucky), were used in the preparation of 10 N KOH, part of the incubation mixture.

Kodak L-(+)-Ascorbic Acid, USP Reagent, (Eastman Kodak, Co., Rochester, New York) was utilized for preparation of 25% solutions that were also part of the incubation mixture.

Isotonic Buffered Saline (American Scientific Products, Mc Gaw Park, Illinois) was added to the cellular suspensions and for sample preparation for the incubation mixture. The buffered saline contained sodium chloride 8 g/L, potassium chloride 0.38

g/L and 1.0 g/L sodium azide. The pH of the isotonic saline was 7.45 as determined by a Corning Blood Gas Analyzer.

The ethyl alcohol used in preparation of samples and standard reconstitution was USP Absolute-200 Proof (Aaper Alcohol and Chemical Co., Shelbyville, Kentucky).

B.) Standards

The following standards were used in the experiment: alpha-tocopherol, beta-tocopherol, gamma-tocopherol, and delta-tocopherol. Alpha, beta, and gamma-tocopherol were all supplied by Supelco, Inc., Bellefonte,Pa. Delta-tocopherol was donated by Dr. Peter Sorter, Hoffman LaRoche, Inc., Natley, NJ.

C.) Apparatus

The High Performance Liquid Chromatographic instrument, IBM Model LC/9533 was purchased from IBM Instruments, Inc., Danbury, CT. A block diagram of the instrument is shown in Figure 5.

The column used was a Radial-Pak Liquid Chromatography Cartridge, C-18, with the following specifications: length 11.9 cm, packing length 10.0 cm, OD 13 mm, ID 8 mm, and particle size of 5 microns. The column was protected by a guard column that also contained octadecylsilane. Both were purchased from Waters Associates, Inc., Milford, MA. The column is contained in a Radial Compression Module-RCM 100 manufactured by Millipore Corporation Waters Chromatography Division, Milford, MA.

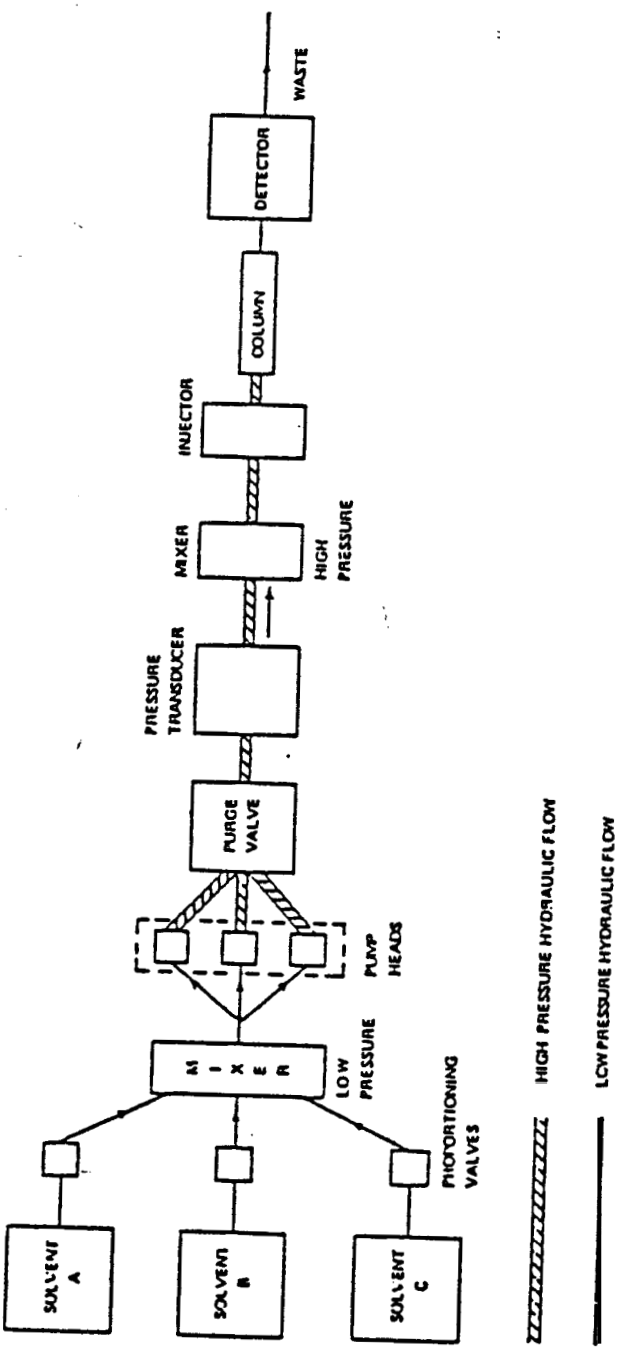


Fig. 5 : Block Diagram of LC/9533

The Millipore Filtration Apparatus (1000 mL size) (Millipore Corporation, Bedford, MA.) was used for the filtration of all samples. Nylon 66 filters of 0.45 µm size and 47 mm diameter were used for solvent filtration.

A Microliter Syringe (Rainer Instruments Co., Inc., Woburn, MA.) of the 500 µl size was used for injection of samples into the HPLC.

The incubation mixture was kept at 70°C in a GCA/Precision Scientific Co. Shaker Bath Model 25 (GCA Corporation, Chicago,IL.)

Centrifugations were done with a GLC-1 centrifuge by Sorvall (Sorvall, Newton, CT.).

The pH determinations on the isotonic saline were made with a Corning 175 pH/Blood Gas Analyzer (Corning Medical and Scientific, Medfiold, MA.).

Hematocrit determinations of patient cello were accomplished with a Coulter Model S-Plus V, Coulter Electronics, Inc., Hialeah, FL.

Fluorescent detection was made possible with a Shimadau Fluorescence HPLC Monitor Model RF-530 (Shimadzu Corp. Spectrophotometric Inst. Plant Analytical Inst. Division, -- Kjoto, Japan).

A vortex mixer was used for the mixing of all samples. It was purchased from Scientific Industries, Springfield, MA.

A vacuum pump, Model 5KH32EG550, was used to degass the mobile phase and was purchased from Sargent-Welch Scientific Co., Skokie, IL.

Data acquisition was via a Fisher Recordall Chart Recorder (Fisher Scientific Co., Pittsburgh, Pa.) and via an IBM 9000 Computer (IBM Instruments, Inc., Danbury, CT.)

CHAPTER IV

EXPERIMENTAL METHODS

A. Preparation of Mobile Phase

Various procedures utilized combinations of methanol and water or 100% methanol as the mobile phase. A mobile phase of 100% methanol yielded the shortest retention times for the isomers of tocopherol. However, a volume ratio of 99:1 (methanol:water) yielded better resolution of the isomeric forms with only a slightly longer retention time for each isomer.

Ultrafiltered HPLC grade methanol and deionized water were thoroughly mixed in the proper ratio. The mobile phase was then transferred to a one liter bottle which was provided in the solvent tray of the LC/9533. The solvent was then degassed using a vacuum pump and a gaseous layer of Helium was placed over the solvent at a pressure of 5 lb/in^2.

B. Preparation of Solutions

Ascorbic acid (25%) was made by dissolving 6.25 mg of the powder in deionized water using a 25 mL volumetric flask.

The potassium hydroxide (10 N) was prepared by dissolving 250 g in 100 mL of deionized water.

The alpha, beta, and gamma standards of 50 mg/mL were made into stock and working standards as follows:

0.5 mL of concentrated standard was dissolved in 50 mL ethanol for stock standard concentration of 0.5 mg/mL . Then, 0.5 mL of the stock standard was dissolved in 50 mL ethanol for working standard concentrations of 0.005 mg/mL or 5 µg/mL. These were then stored at 4°C in volumetrics protected fran light by aluminum foil.

Delta-tocopherol was prepared by dissolving 20 mg of this vitamin in 1 mL of ethanol to produce a concentration of 20 mg/mL. One-half of a milliliter of this was then diluted to 50 mL to make a stock standard of 0.20 mg/mL or 200 µg/mL. The working standard war prepared by diluting 5 mL of the stock standard to 50 mL for a concentration of 20 µg/mL. This standard was also stored at 4°C and protected from exposure to light.

C. Preparation of Samples Prior to Analysis

EDTA preserved whole blood is collected from a human subject. A volume of 5 mL of whole blood is acceptable for analysis. (As little as 3 mL may be used if the subject is a pediatric patient or procurement of the specimen is difficult.) The specimen Should be thoroughly mixed following phlebotomy to insure distribution of the anticoagulant. The specimen should then be centrifuged to separate the cellular and plasma layers. The plasma is subsequently removed and a volume of isotonic saline added. The cellular elements should then be resuspended, recentrifuged, and the isotonic saline removed. This step should be repeated once more to insure complete removal of the plasma

from the cells. At this point, a volume of isotonic buffered saline is added so that the hematocrit is approximately 50%, as verified by a Coulter Model S Plus V Hematology Analyzer. The cellular suspension can then be frozen for up to four weeks at -20°C. Caution should be exercised at all times to protect the samples from unneccessary exposure to light, as vitamin E is light sensitive.

D.) Preparation of Incubation Mixture

Preparation of the incubation mixture was based upon the work done by Chiu and Lubin (28). Table 2 gives the volumes and concentrations of the components used in the incubation mixture.

Initially, 2.0 mL of isotonic saline is added to a 15 mL glass test tube labeled appropriately as blank, standard, or patient. To this, 1.0 mL of either, H_2O, standard, plasma or cell suspension was added and thoroughly mixed. At this point, 0.5 mL of freshly prepared ascorbic acid and 1.0 mL of either water or ethanol (as indicated for patient or standard) are added. Ascorbic acid prevents oxidation of tocopherol into tocopherol quinone.

The mixtures are then stoppered and placed in a 70°C reciprocating water bath for 5 minutes. At the completion of this time, all test tubes are removed and 1.0 mL of 10 N KOH is added, the mixtures vortexed, restoppered, and returned to the water bath for an additional 30 minutes at the same temperature.

TABLE 2

Volume (mL) of Components in Incubation Mixture

	Saline	H_2O	Ascorbic Acid	KOH	Ethyl Alcohol	Sample*	Total
Blank	2.0	1.0	0.5	1.0	1.0	----	5.5
Standard	2.0	1.0	0.5	1.0	---	1.0	5.5
Patients (C or P)**	2.0	---	0.5	1.0	1.0	1.0	5.5

* Sample= Cells, Plasma, or Standard

** Cells or Plasma

When the incubation is completed, the samples are removed and cooled for 10 minutes. Following cooling, 2.0 mL of hexane is added to each test tube, and the solutions are vortexed for 1 minute to insure extraction of the tocopherol into the hexane layer. All test tubes are then centrifuged for 10 minutes to separate the hexane layer. After centrifugation, the hexane layer was removed to a separately labeled tube prior to injection for ease of analysis.

E.) HPLC Method

The experiment used the IBM Model LC/9533 (Figure 5). A Waters C_{18} Reversed Phase Column preceded by a guard column was used. Both columns were maitained at room temperature. The mobile phase consisted of methanol:water (99:1, v/v). The column inlet pressure and guard column pressure were 55-70 bars. The flow rate was 1.6 mL/minute. A Shimadzu Fluorescent Monitor was used for fluorescent detection. Excitation wavelength was at 290 nm and emission wavelength at 330 nm. A 100 µL full volume injection loop was used, and an excess of 100 µL was injected in accordance with the full loop injection technique (29).

F.) Data Acauisition and Manipulation

Acquisition of data was via a Fisher chart recorder and an IBM 9000 Computer equipped with a Chromatography Applications Program version 1.4. The chart recorder served primarily as a

back-up for the IBM 9000, and primary data storage and manipulation was via this computer.

The following pages (37-40) are the method pages utilized by the IBM 9000 for data accumulation as used in this research.

The IBM 9000 also allows the user to reintegrate the raw data via a reintegration mode. Page 41 shows a portion of the program that directs this function.

Various other screens are auailable for the user to implement. The following pages detail same of these added functions.

Page 42 shows the screen that appears when one is ready to make a sample run and to accumulate data that is to be saved to an appropriately identified data diskette.

Page 43 shows the status of the channels at any given time, displays run times, minutes elapsed since beginning of run, and the total number of runs desired.

Page 44 shows the final screen available once the system is shutdawn.

Page 45 details the screen that aids the user tomaks a copy of a diskette.

Page 46 is a copy of the calibration table used in-this research . An external standard was prepared and run with each sample run and was compared at intervals to the standard calibration table.

REDCELL METHOD IDENTIFICATION

Page

>1 Title
2 Acq'n
3 Events
4 Baseln
5 Calib
6 Names
7 Report

Std %

1 1:C1D

1 Name [REDCELL]:-
2 Author [DARLENE VASBINDER]:
3 Instrument [LC/9533]:
4 Column [WATERS]:
5 LC Procedure disk file name [DAR]:
6 Notes []

TUE 26 MAY 87 19:28:12

LINE PAGE CONC EXIT

REDCELL

ACQUISITION TIMED EVENTS

"P": Peak detect [P]
"M": Negative peaks [-M]
"I": Inhibit end of peak [-I]
"-": Turn off next event code
"0.. 9, A. .F": External events

Page

1 Title
2 Acq'n
>3 Events
4 Baseln
5 Calib
6 Names
7 Report

Std #

Time	Event codes
0.00	-P
6.50	P

TUE 26 MAY 87 19:27:54

PAGE CONC OMIT . <— EXIT

```
REDCELL          BASELINE CORRECTION TIMED EVENTS
                   "E": Xponential skim [E]
 Page              "F": Force baseline at next valley [-F]
 ----              "H": Force horizontal baseline [-H]
 1 Title           "V": Farce baseline at every valley [-V]
 2 Acq'n           "-": Tarn off next baseline code
 3 Events
>4 Baseln          Time       Baseline codes
 5 CalID          -
 6 Names
 7 Report

 Std #
 ----
 1 1:CID
```

TUE 26 MAY a7 19:23:59

PAGE CONC OMIT <— EXIT

REDCELL NAMED PEAKS LIST

Page	Retn time	Peak name	Group	Ref/Std
1 Title	9.00	GAMMA	0	
2 Acq'n	18.48	ALPHA	0	
3 Events				
4 Baseln				
5 Calib				
>6 Names				
7 Report				

Std #

1 1:C1D

TUE 26 HAY 87 19:24:51

PAGE CONC OMIT <—— REFPK STDPX WIT

PREPARE REINTEGRATION WITH METHOD NAME REDCELL

1 METHOD ORIGIN [DAR]:
2 DATA FILE NAME (1-15 CHRS.) [1:C1DS0028]:

3 NOTES []
: _

4 CONVERSION FACTOR [1.000E+00]:

5 VOL. INJECTED (ul) [100.0]:
6 SAMPLE MOUNT [1.00]:

TUE 26 MAY 87 19:15:08

LINE EXIT

-

READY CHANNEL #1 WITH METHOD NAME REDCELL

---

1 METHOD ORIGIN [DAR]:
2 SAMPLE NAME [STD]:
3 DATA SAVE MODE (N,R,P,H,A) [A]:
DATA DISX ID (1-6 CHRS.) [DATA]:
DATA FILE NAME (5 CHRS.) [C1DS]:
DATA PILE SEQ. NUMBER (1-999) [1]:

4 NUMBER OF RUNS DESIRED (1-999) [1]:

5 PLM DESIRED (Y/N) [Y]:
PLOT AREA(1=FS,2=US,3=LS,4=FP,5=RP,6=LP)[1]:_
LOWER PLM LIMIT (-5 to 999mv) [0]:
UPPER PLOT LIMIT (-4 to 1000mv) [100]:
PLOT RATE (may use soft keys) [1.00]:

6 NOTES [I

7 CONVERSION FACTOR [1.000E+00]:

8 VOL. INJECTED (ul) [100.0]:
9 SAMPLE AMOUNT [1.00]:

TUE 26 MAY 87 19:12:42

Screen Plotting rates in cm/min

LINE 8.25 8.50 1.88 3.88 5.88 9.80 EXIT

CURRENT ACTIVITIES

CHAN #	INPUT VOLT	METHOD NAME	DATA FILE NAME	RUN #/ TOT RUNS	PLOT AREA	STATE	MIN. ELAPSED/ RUN TIME
#E		PRESET					
0		PRESET	C0DNS			IDLE	
1	1	PRESET	C1DNS	1	FS/IDLE	IDLE	0.00/ 38.88
2	1	PRESET	C2DNS	I	FS/IDLE	IDLE	0.00/ 30.80
3	10	PRESET	C3DNS	1	FS/IDLE	IDLE	0.00/ 38.80
4	1	PRESET	C4DNS	1	FS/IDLE	IDLE	0.00/ 38.08
#L			LC/9533 CURRENT STATUS				

CH Pr# Time Flow SolA SolB SolC

CMD>

TUE 26 MAY 87 19:11:11

COPY DATE. HELP RENAME CONFIG

EXIT TO THE OPERATING SYSTEM SHUTS DOWN ALL CHANNELS.
ALL DATA AND METHODS HOT SAUED ON DISK WILL BE LOST.

DO YOU WANT TO EXIT?[N]_ TOE 26 MAY 87 19:29:18

Y N

```
DISXCOPY
CS/9000  DISKCOPY  1.1     8/29/83
COPYRIGHT (c) 1982, 1983 IBM INSTRUMENTS, INC.
ENTER SOURCE DRIUE NUMBER:  0
ENTER DESTINATION DRIUE NUMBER:  1
ENTER OPTIONS:  V
WARNING:  DESTINATION DISK CONTENTS
          WILL BE DESTROYED
COPY FROM DRIUE 0 TO DRIUE 1 ? Y
CURRENT VOLUME IDENTIFIER IS:  DATA
CHANGE DESTINATION VOLUME IDENTIFIER (Y/N) ? N
DISKCOPY COMPLETED
_
```

REDCELL CALIBRATION STANDARD LEVEL 1 "1:C1DS017"

Page	Line	Time	Peak name	Amount	Area	R-factor
1 Title	> 1	9.88	GAMMA	6.25E-01	15791	2.53E+02
2 Acq'n	2	10.40	ALPHA	2.50E+00	38876	1.56E+02
3 Events						
4 Baseln						
5 Calib						
6 Names						
7 Report						

Std #

>1 1:C1D

THU 04 JUN 87 14:09:25

LINE PAGE COHC OMIT <— —> RFFUNCTN EXIT

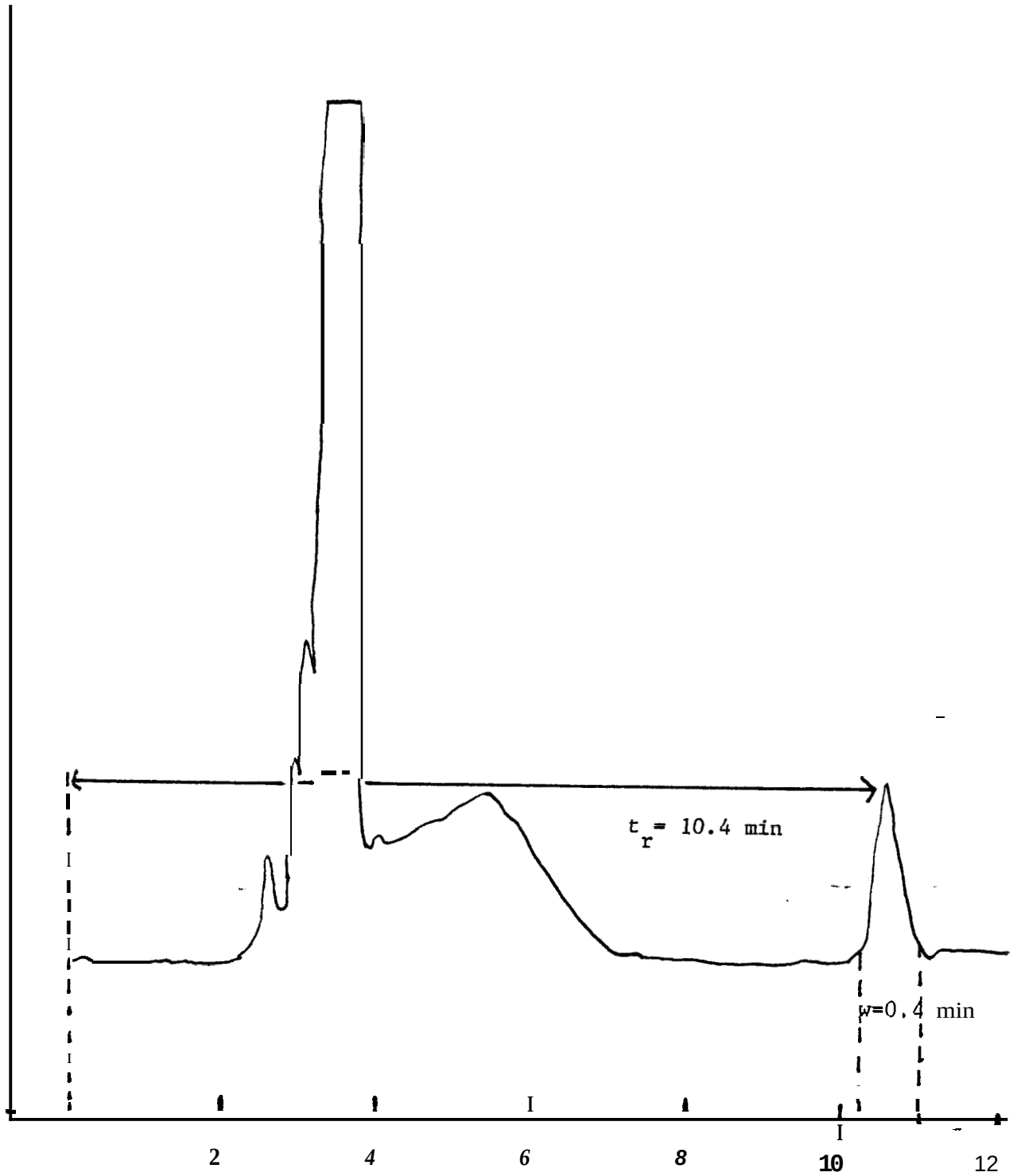
CHAPTER V

RESULTS AND DISCUSSION

Success with High Performance Liquid Chromatography depends upon the efficiency of the column and the mobile phase used. The C_{18} Radial-Pak Liquid Chromatography Cartridge was purchased from Waters Associates, Inc., and was evaluated for efficiency by calculating the number of theoretical plates in accordance with Equation 2, reshown below. The chromatogram on the following page (Fig. 6) shows the values used in this calculation and how they were obtained. The resultant value of 10,816 agrees well with the manufacturer's limits of 10,000±10%. Therefore, this calculation demonstrated the efficiency of this column.

$$N = 16\left(\frac{t_r}{w}\right)^2 = 16\left(\frac{10.4}{0.4}\right)^2 = 10,816 \qquad (2)$$

All samples and standards were analyzed using this column, which had a particle size of 5 microns. The procedures followed by Cook (21), Taylor et al (20), and Chiu and Lubin (13) were modified and used in this research. The following is a description of these changes: Taylor's method was modified so that the saponification mixture contained 2.0 mL of phosphate



Time in Minutes

Fig. 6. Chromatogram Utilized in Calculating Column Efficiency

buffered saline, 0.5 mL of 25% ascorbic acid, 1.0 mL of 100% ethanol and 1.0 mL of rbc's or plasma. Taylor's group also used a neutral alumina column to which the hexane extract was applied, whereas this researcher selected a C_{18} Bondapak column. Chiu and Lubin added a 4.0 mL volume of hexane for extraction, as did Taylor. This was decreased to a 2.0 mL quantity in order to improve assay sensitivity. In addition, Chiu utilized heparinized samples, whereas, due to accessibility, EDTA samples were used in this assay. Cook performed her analysis with the same column as was used in this procedure. Selection of this column over others was made on the basis of possibly improving reproducibility and reliability and canplete elimination of column voiding and channeling (30). A decision for flow rate of the solvent was based upon a canparision of 1.0 mL/minute, 1.6 mL/minute and 2.0 mL/minute. The first rate yielded too long of a retention time for the isaneric canponents, whereas, the last did not allow sufficient time for return to baseline following the solvent front. Therefore, the intermediate time of 1.6 mL/minute was selected.

A solvent ratio of 99:1 (methanol/water) was chosert-for this procedure, as it allowed good resolution of the isomeric peaks. The following formula shows this factor as used with the values in the chromatogram on page 51.

$$R = \frac{2\Delta t}{w_a + w_b} \qquad (5)$$

R = resolution

t = difference in retention times

w_a, w_b = width of peaks at their bases

Ideally, resolution should be at least 1.0, and this agreed well with the calculated value of 1.4.

The retention times of alpha–,gamma–,beta–,and delta–tocopherol standard are shown in Table 3. The order of the retention times corresponds well with the anticipated relationship based on the structures of the tocopherol isomers.

TABLE 3
Retention Times of Compounds Investigated in This Assay

Compound	Retention Times (Minutes)
Alpha–tocopherol	10.4
Beta–tocopherol	9.4
Gamma–tocopherol	9.4
Delta–tocopherol	8.0

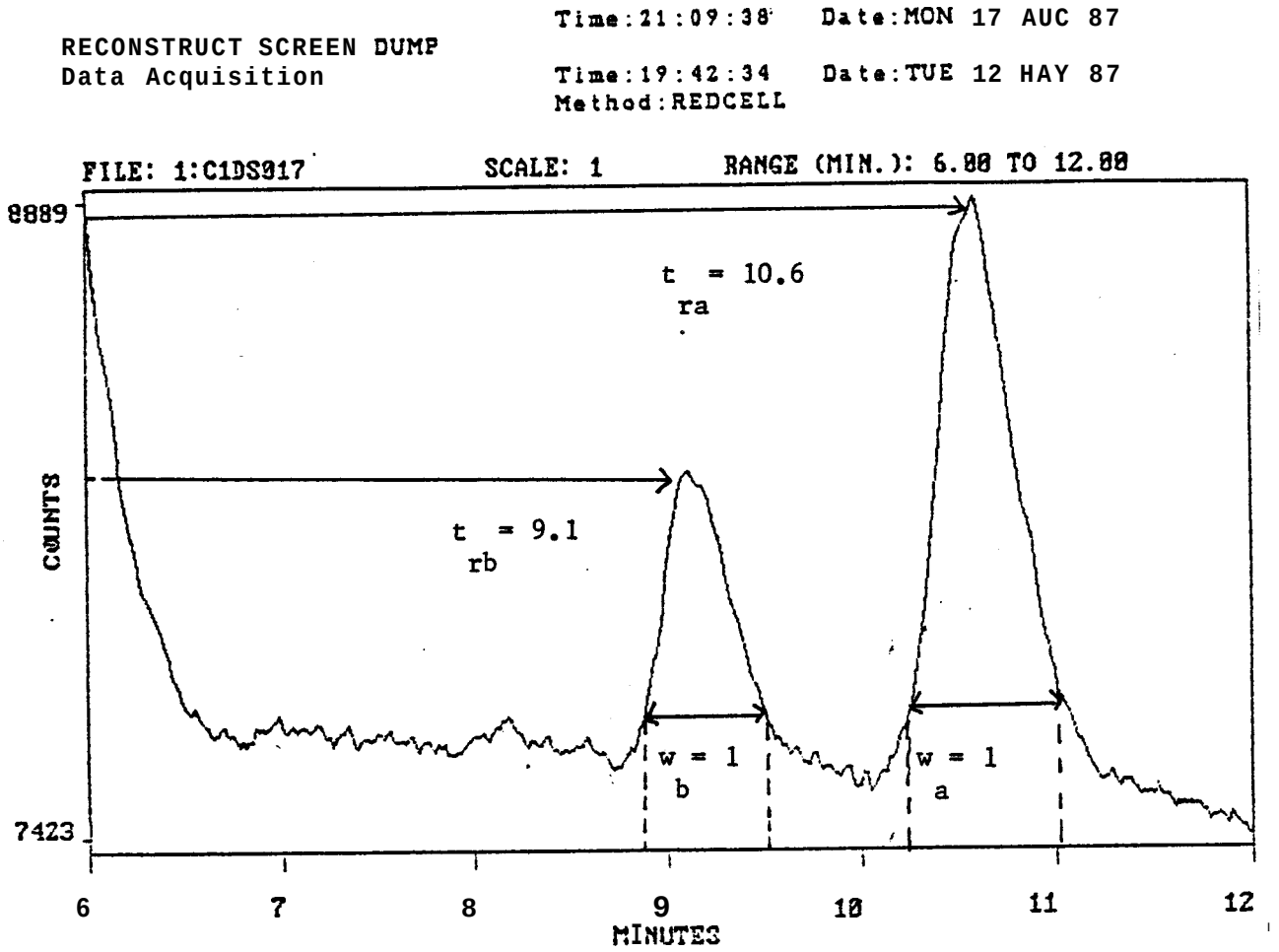


Fig. 7. Chromatogram Utilized in Calculating Resolution

Once the desired retention times were determined, a standard curve for each isomeric form was constructed. Values for peak areas were calculated by the IBM 9000 computer and plotted versus concentration in µg/mL. Tables 4–7 show the peak areas per isomeric form. The standard curves for each isomer are presented in Figures 8–11. The standard curve dilutions were chosen to cover the range expected to be found in normal patients red cells and plasma. The standard curve plots are done by linear regression analysis. Table 8 contains the formulae utilized in the calculation of each isomers' standard curves.

Since this procedure required extensive sample pretreatment, an external standard was run with each assay and compared to that value anticipated for the standard. The IBM 9000 allows the user to calculate analyte concentrations based upon a response factor and peak area according to the following formula:

Response Factor = peak area or height of sample X amount of isomer in the standard as entered in the Calibration Table

The Calibration Table used in this research was shown on page 46. Further computations were then based upon this standard, which consisted of a mixture of a mixture of alpha and gamma tocopherol (2.5 µg/mL and 0.625 µg/ml, respectively).

Since beta coelutes with gamma, it was not included in the standard mixture. It then follows, that the gamma tocopherol concentrations reported include beta. Delta was also excluded from the standard mixture , but for a different reason. All

TABLE 4

PEAK AREAS OF ALPHA-TOCOPHEROL STANDARD

Alpha-tocopherol Concentration (µg/mL)	Peak area
25.0	397498
12.5	240747
5.0	78193
2.5	39118
1.25	15667
0.625	6931
0.312	3210

TABLE 5

PEAK AREAS OF BETA-TOCOPHEROL STANDARD

Beta-tocopherol Concentration (µg/mL)	Peak Area
5.0	170620
2.5	78006
1.25	50570
0.625	35913
0.312	17985

TABLE 6

PEAK AREAS OF GAMMA-TOCOPHEROL STANDARD

Gamma-tocopherol Concentration (µg/mL)	Peak Area
5.0	144165
2.5	82434
1.25	49808
0.625	18590
0.312	7333
0.15	4509
0.075	1243

TABLE 7

PEAK AREAS OF DELTA-TOCOPHEROL STANDARD

Delta-tocopherol Concentration (µg/mL)	Peak Area
4.0	137300
2.0	80710
0.50	23600
0.250	14400
0.125	11100

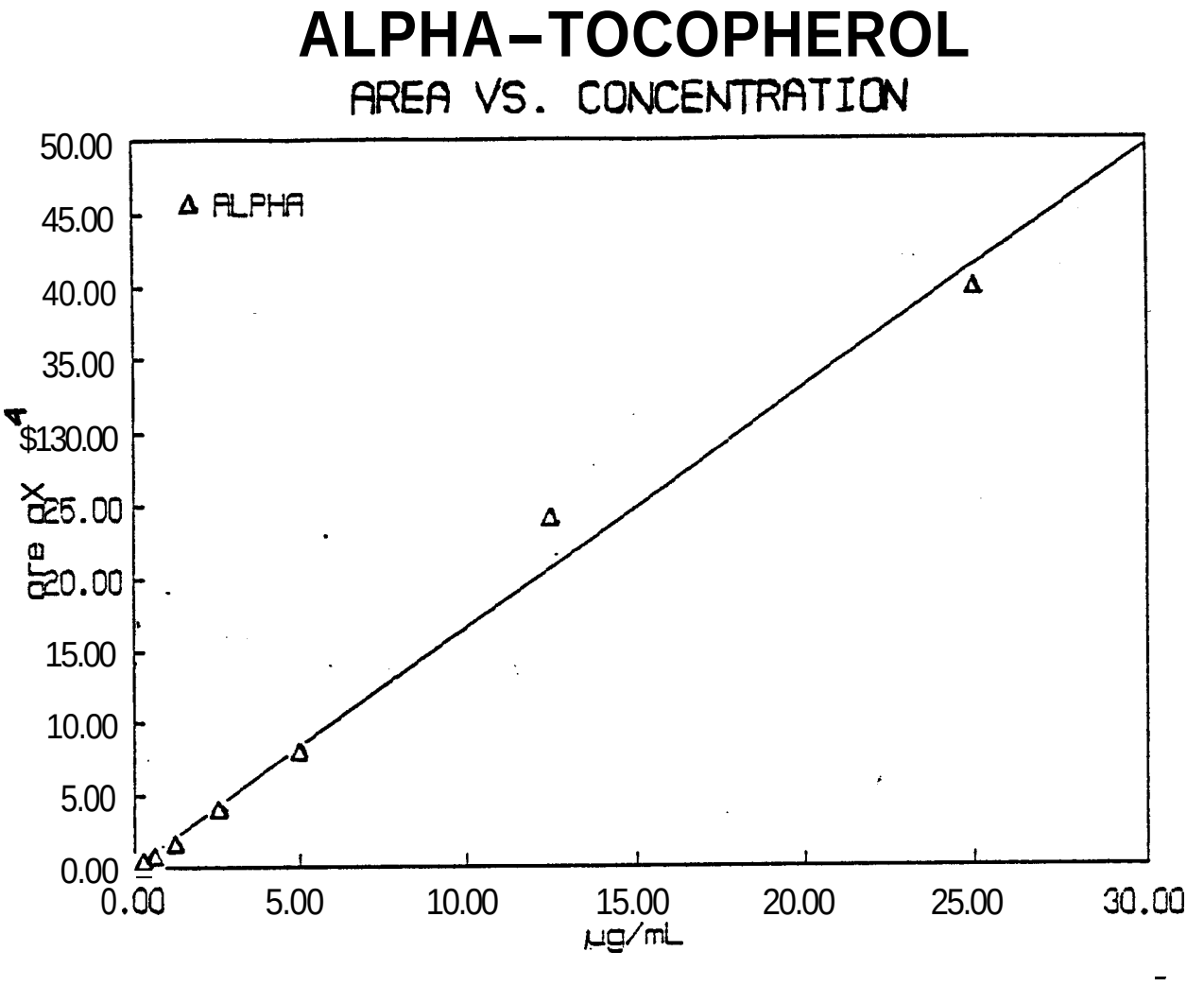


Figure 8. Alpha-tocopherol Standard Curve

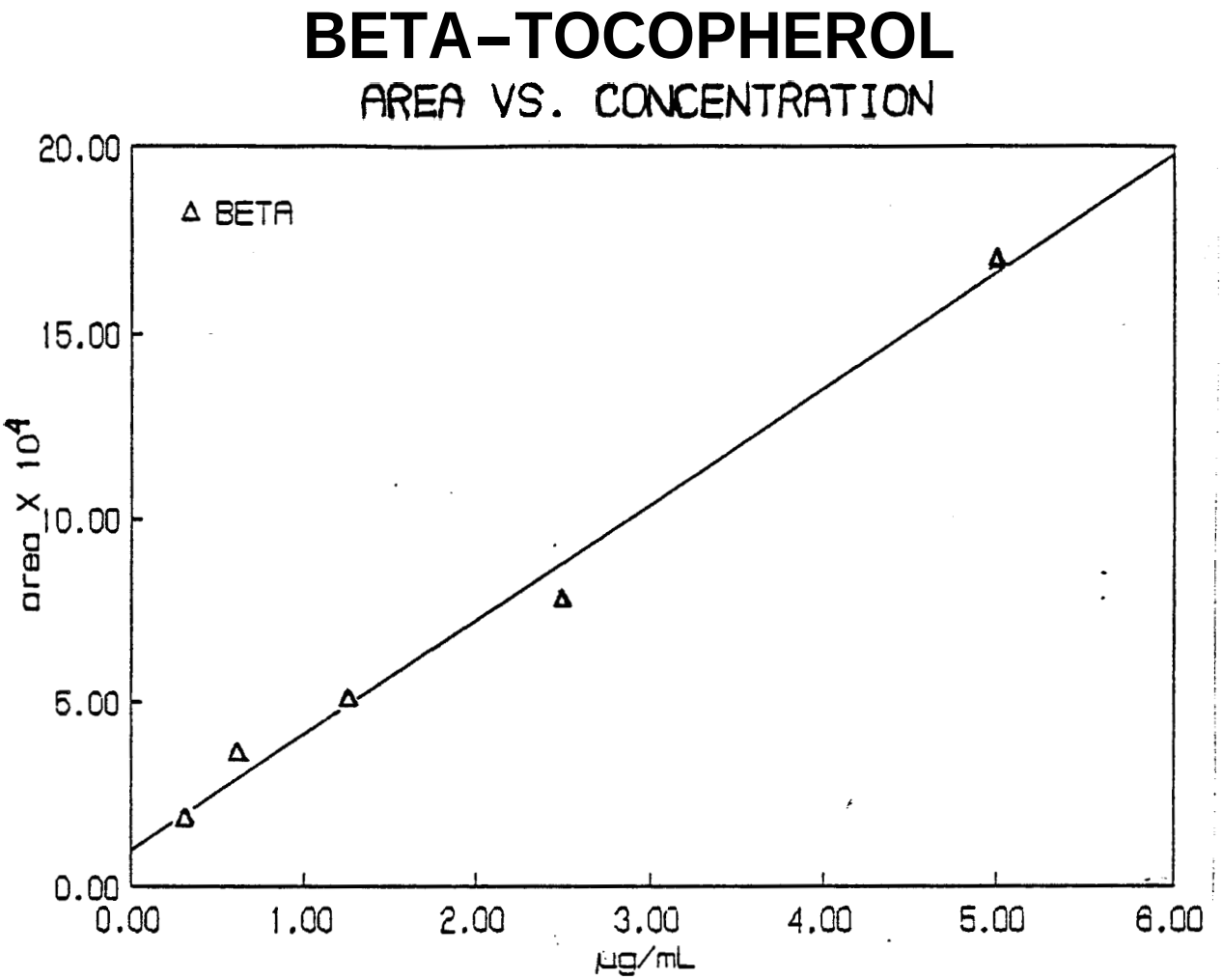


Figure 9. Beta-tocopherol Standard Curve

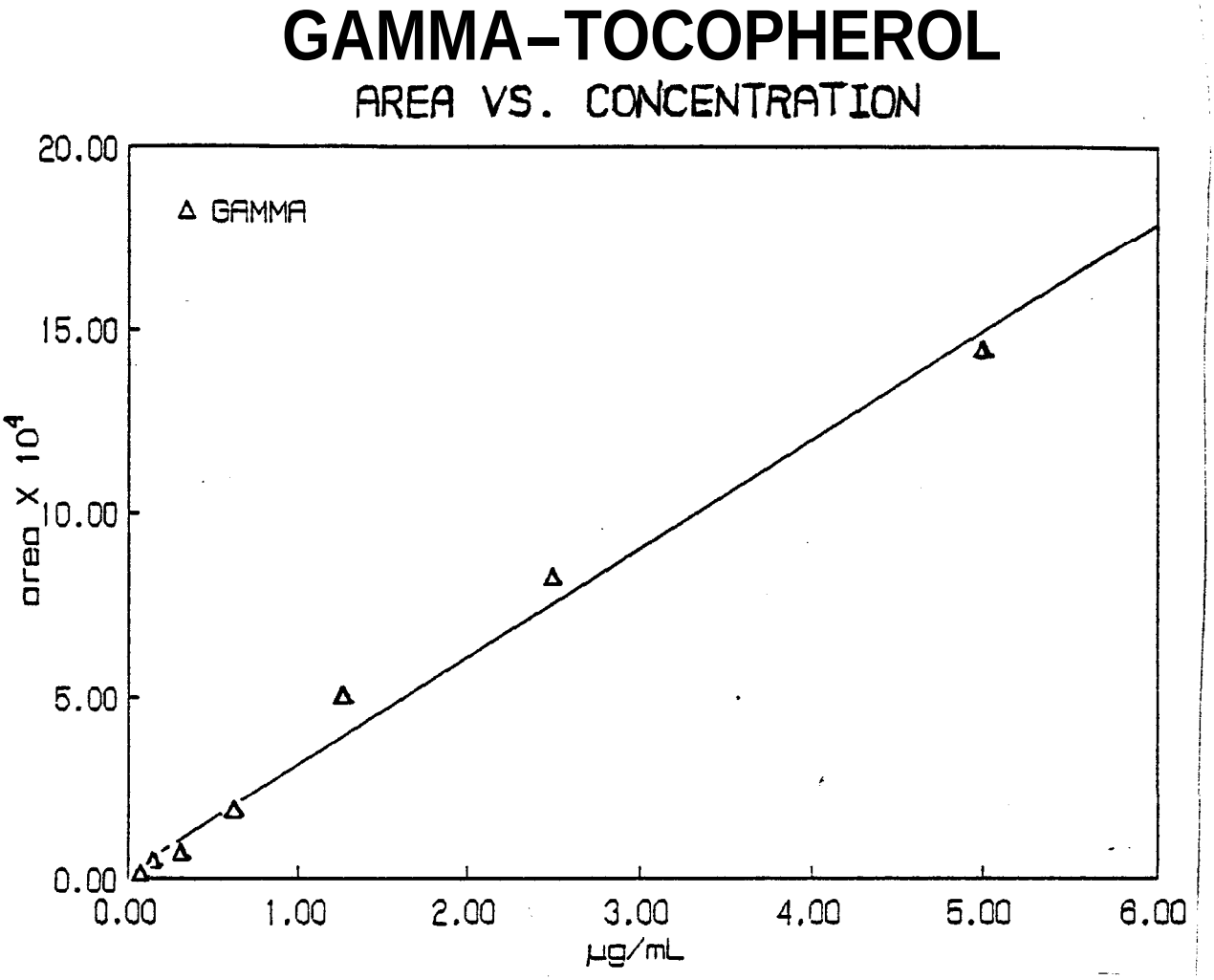


Figure 10. Gamma-tocopherol Standard Curve

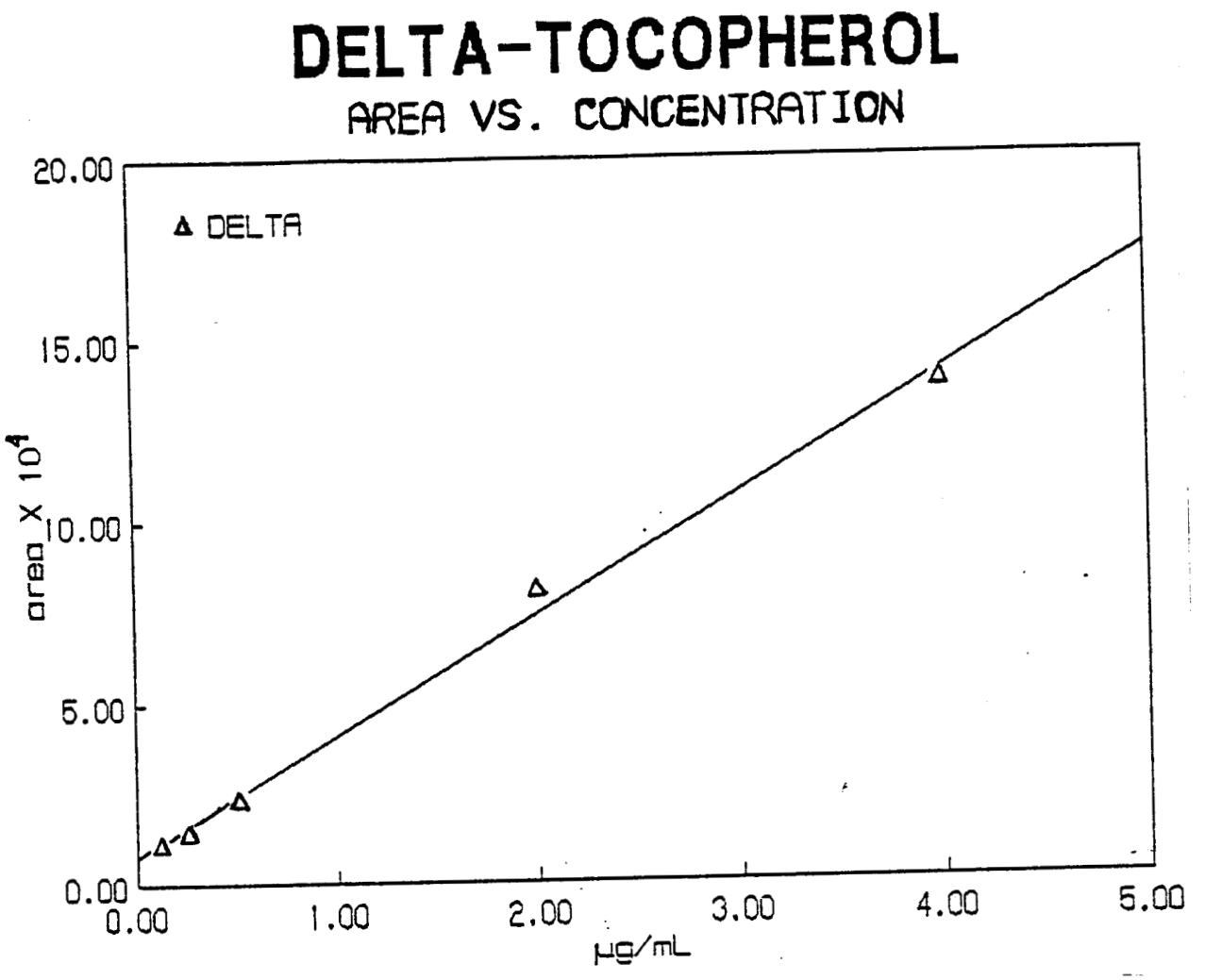


Figure 11. Delta-tocopherol Standard Curve

TABLE 8

Y ON X REGRESSION EQUATIONS FOR STANDARD CURVES

alpha	Y = 0.03231 + 49.51514 x ((X - 0.00)/30.0)
beta	Y = 0.98939 + 18.80028 x ((X- 0.00)/6.0)
gamma	Y = 0.23398 + 17.60000 x ((X-0.00)/6.0)
delta	Y = 0.78870 + 16.55747 x ((X- 0.00)/6.0)

isomers in the standard mixture must be detected in samples in order for the IBM 9000 to generate an External Standards Report with ug/mL values (Figure 12). If one of the isomers is not found, an area percent report is generated (Figure 13). Due to the low concentration of delta-tocopherol, it was decided to delete delta from the standard mixture. Peaks subsequently identified as delta were then quantitated based on a simple ratio of peak area to concentration.

Normal individuals were analyzed for alpha-, gamma-, and total tocopherol in both their plasma and red blood cells. The raw data of these assays is shown in Table 9. Table 10 represents the erythrocyte tocopherol levels of an additional ten normal individuals. The mean, standard deviation and standard error for the alpha-, gamma-, and total tocopherol of these patients is shown in Table 11 . These calculations were based on the raw data from Table 9 only. Table 12 shows Confidence Limits on the Mean for both normal individuals and Sickle Cell patients at the 90,95, and 99% intervals.

Table 13 displays the raw data from patients homozygous for Hemoglobin S. Seven patients' cells were analyzed and of these, five plasmas were available for tocopherol determination. The mean, standard deviation and standard error of these data are also shown in Table 11.

Various correlation matrices are shown in Tables 14-17. A brief explanation for the interpretation of these (r) values follows (31):

Channel #.....REINT Time:17:36:27 Date:TUE 02 JUN 87

Sample name.....STDS
Data file.....1:C1DS017
Method name.....REDCELL

Run time.....12.00 min. Delay time...0.00 min.

Area reject....0
peaks found..2
Vol. Inj.....100.0 ul Sample amount..1.000
Conv. factor...1.000

EXTERNAL STANDARDS REPORT

Peak.	R.T.(min)	Ref	Peak name	UG/ML	Area	BL
1	9.115		GAMMA	6.250E-01	15791	BB
2	10.598		ALPHA	2.500E+00	38876	BB
TOTALS				3.125E+00	54667	

Figure 12. IBM External Standards Report

Channel #.....REINT Time:17:35:06 Date:TUE 02 JUN 87

Sample name.....STDS
Data file.....1:C1DS017
Method name.....REDCELL

Run time.....12.00 min. Delay time...0.00 min

Area reject....0
peaks found..2

NORMALIZATION REPORT

Peak	R.T.(min)	Ref	Peak name	Norm %	Area	BL
1	9.115		GAMMA	20.000	15791	BB
2	10.598		ALPHA	80.000	38876	BB
TOTALS				100.000	54667	

Figure 13. IBM **Normalization** Report

TABLE 9

RAW DATA FOR CELLS AND PLASMA ALPHA-,GAMMA-, AND TOTALTOCOPHEROL(μg/mL)
NORMAL INDIVIDUALS

Cell			Plasma		
Alpha	Gamma	Total	Alpha	Gamma	Total
0.732	0.221	0.953	10.570	2.004	12.570
1.219	0.336	1.555	9.269	2.261	11.530
1.522	0.403	1.925	9.122	2.168	11.290
1.135	0.103	1.238	12.810	1.285	14.100
1.190	0.262	1.452	17.830	3.567	21.390
0.935	0.635	1.570	5.511	4.114	9.625
1.123	0.040	1.163	6.138	1.120	7.258
0.773	0.357	1.130	2.930	0.823	3.753
1.019	0.352	1.371	8.968	1.214	10.180
1.025	0.112	1.137	7.791	1.027	8.818

TABLE 10

RAW DATA FOR RED CELL LEVELS OF ALPHA-,GAMMA-,AND TOTAL TOCOPHEROL(μg/mL) FOR AN ADDITIONAL 20 NORMAL INDIVIDUALS

Alpha	Gamma	Total
1.118	0.342	1.460
0.615	0.337	0.952
0.894	0.227	1.121
1.090	0.171	1.261
1.372	0.411	1,783
0.863	0.383	1.246
1.032	0.368	1.400
0.663	0.262	0.925
0.983	0.489	0.472
1.133	0.103	1.236
1.377	0.274	1.651
0.732	0.221	0.953
1.219	0.336	1.555
1.522	0.403	1.925
1.135	0.103	1.238
1.190	0.262	1.452
0.935	0.635	1.570
1.123	0.040	1.163
0.773	0.357	1.130
1.019	0.352	1.371
1.025	0.112	1.137

TABLE 11

TOCOPHEROL LNELS (MEAN ± S.D.) NORMAL AND SICKLE CELL

	Alpha	Gamma	Total
Normal Individuals-Cells(all raw data)			
mean±S.D.	1.07±0.23	0.28±0.18	1.35±0.29
standard error	0.07	0.55	0.09
Normal Individuals-Plasma(all raw data)			
mean±S.D.	9.09±4.13	1.96±1.12	11.05±4.65
standard error	1.31	0.35	1.47
Sickle Cell Patients Cells			
Mean±S.D.	1.22±0.33	0.68±0.29	1.91±0.37
standard error	0.12	0.11	0.14
Sickle Cell Patients Plasma			
Mean±S.D.	5.84±0.32	3.40±1.33	9.24±1.31
standard error	0.14	0.60	0.59
Normal Individuals Plasma(recalculated data)*			
Mean±S.D.	8.77±2.34	1.90±1.02	10.67±2.17
standard error	0.83	0.36	0.77

*Data evaluated with 2.5d rule (38) and rejected accordingly

TABLE 12

CONFIDENCE LIMITS ON THE MEAN (99,95, and 90% LOWER AND UPPER LIMITS) FOR NORMAL INDIVIDUALS' AND SICKLE CELL PATIENTS'

		99		95		90	
Normal							
Cell	alpha	0.83	1.30	0.90	1.23	0.93	1.20
	gamma	0.10	0.46	0.16	0.41	0.18	0.38
	total	1.06	1.64	1.15	1.55	1.18	1.52
Plasma	alpha	4.85	13.34	6.14	12.05	6.70	11.49
	gamma	0.81	3.11	1.16	2.76	1.31	2.61
	total	6.28	15.83	7.73	14.38	8.36	13.75
Sickle Cell							
Cell	alpha	0.76	1.68	0.92	1.53	0.98	1.46
	gamma	0.28	1.09	0.42	0.95	0.47	0.90
	total	1.38	2.43	1.56	2.25	1.63	2.18
Plasma	alpha	5.19	6.49	5.45	6.23	5.54	6.14
	gamma	0.66	6.14	1.74	5.06	2.13	4.67
	total	6.55	11.94	7.61	10.87	7.99	10.49

TABLE 13

SICKLE CELL DISEASE PATIENTS'
RAW DATA FOR CELLS AND PLASMA ALPHA-, GAMMA-, AND TOTAL TOCOPHEROL
(μg/mL)

Cells			Plasma		
Alpha	Gamma	Total	Alpha	Gamma	Total
0.87	0.61	1.48	6.30	3.16	9.46
1.09	0.46	1.55	5.87	1.73	7.60
1.74	0.51	2.25	5.43	3.21	8.64
0.95	1.09	2.04	----	----	----
1.56	0.45	2.01	----	----	----
1.02	0.56	1.58	5.72	5.46	11.18
1.32	1.11	2.43	5.89	3.44	9.33

When two variables are linearly independent in that the sense that knowing a value of one variable is of no help in predicting a corresponding value for the other variable, $r = 0$. A positive correlation implies that high values of one variable tend to accompany high values of the other and vice versa. A negative correlation implies that high values of one variable tend to accompany low values of the other (and vice versa). The last two statements suggest that when $r = 0$, values of one variable can be used to predict the values of the other. The accuracy of the prediction (i.e., the strength of the linear relationship) is determined by the size of r. The higher the value of r, the more accurate the prediction. If $r = 1$, (i.e., if $r = 1$ or $r = -1$) prediction is perfect.

Usually r is not zero, nor is it plus or minus one. The relative strength of r is proportional to r, which represents the proportion of the variation (error) in one variable which can be attributed to its linear relationship with the other variable.

The correlation user must remember that r measures only the linear relationship between two variables. Even though r may be small, it is possible that better predictions could be made if a non-linear relationship (curvilinear) were investigated. This researcher opted not to investigate these relationships in this project.

A correlation matrix (Table 14) was performed on the data from Table 9 and included relating plasma and cellular levels of each assayed component in normal individuals. A correlation matrix was also performed for the Sickle Cell disease

TABLE 14

NORMAL INDIVIDUALS' CORRELATION MATRIX FOR ALPHA-, GAMMA-, AND TOTAL TOCOPHEROL CELL AND PLASMA

	gamma-cells	total-cells	alpha-plasma
alpha-cells	-.0259	.7888	,3580
gamma-cells	-----	.5941	-,2634

	gamma-plasma	total-plasma
alpha-cells	,1541	.3556
gamma-cells	,6311	-,0825
total-cells	.5121	.2354
alpha-plasma	.3547	,9744
gamma-plasma	-----	.5557

patient's data, but care should be exercised in interpretation due to the limited number of samples involved. Table 15 shows this matrix. Two final matrices were performed on the plasma and red cell values of five normal individuals versus five Sickle Cell patients. These correlations are shown in Tables 16 and 17.

The ratio of red cell plasma alpha to gamma in both normal individuals and sickle cell patients is shown below:

Normal

Cell 0.21

Plasma 0.22

Sickle Cell

Cell 0.51

Plasma 0.56

An inspection of the raw data generated by the IBM 9000 showed a peak eluted at approximately 8 minutes on several patient samples. I felt that, considering the retention time of delta-tocopherol, this peak could potentially represent this isomeric form. Tahle 18 shows the raw data for these peaks as found in the 10 normal individuals and four of the sickle patients' cells and plasma. The mean and standard deviation-of this data are shown in Table 19.

An evaluation of the method was performed on both pooled cellular and plasma samples. Reproducibility results are shown in Table 20. The table details both with-in run and between-run results. Reproducibility studies reflect the running of three separate samples each day for three consecutive days and analyzing

TABLE 15

SICKLE CELL PATIENTS' CORRELATION MATRIX FOR ALPHA-, GAMMA-, AND TOTAL TOCOPHEROL CELL AND PLASMA

	gamma -cells	total-cells	alpha-plasma
alpha-cells	.0838	.8090	-.8170
gamma-cells	-----	,6536	.2022
total-cells	-----	-----	-.5012

	gamma-plasma	total-plasma
alpha-cells	-.1137	-.3123
gamma-cells	,1327	.1838
total-cells	-.0081	-.1288
alpha-plasma	-.1947	.0422
gamma-plasma	-----	.9718

TABLE 16

CORRELATION MATRIX OF NORMAL INDIVIDUALS' VERSUS SICKLE CELL PATIENTS' PLASMA TOCOPHEROL LEVELS

	gamma-normal	total-normal	alpha-sickle
alpha-normal	-.0725	,9583	.0642
gamma-normal	-----	.2157	-.0279
total normal	-----	-----	,0538

	gamma-sickle	total-sickle
alpha-normal	.6869	.7151
gamma-normal	-.6664	-.6855
total-normal	.4816	.5035
alpha-sickle	-.1947	.0422
gamma-sickle	-----	.9718

TABLE 17

CORRELATION MATRIX OF NORMAL INDIVIDUALS' VERSUS SICKLE CELL PATIENTS' RED CELL TOCOPHEROL LEVELS

	gamma-normal	total-normal	alpha-sickle
alpha-normal	.0256	,7836	.8038
gamma-normal	-----	.6411	.0611
total-normal	-----	-----	.6550

	gamma-sickle	total-sickle
alpha-normal	-.1162	.6177
gamma-normal	-.7060	-.4934
total-normal	-.5280	.1675
alpha-sickle	-.2755	,6669
gamma-sickle	-----	.5325

TABLE 18

RAW DATA FOR CELLS AND PLASMA DELTA-TOCOPHEROL (µg/mL)

Cell	Plasma
Normal Individuals	
0.050	0.011
0	0.013
0	0
0	0.007
0.008	0.022
0.060	0.093
0.027	0
0.049	0.038
0.015	0.007
0.010	0.043
Sickle Cell Patients	
0	*0.080*
0.060	not available
0.015	0.025
0.027	*0.099*

TABLE 19

MEAN AND STANDARD DEVIATION OF DELTA-TOCOPHEROL FOR NORMAL INDIVIDUALS AND SICKLE CELL PATIENTS
(μg/mL)

Normal Individuals	
Cells	0.0219 + 0.0231
Plasma	0.1234 + 0.3092
Sickle Cell Patients	
Cells	0.0255 + 0.0255
Plasma	0.0680 + 0.0384

TABLE 20

REPRODUCIBILITY OF TOCOPHEROL ANALYSIS

	Alpha-tocopherol(μg/mL) Mean ± S.D.	CV%	Gamma-tocopherol(μg/mL) Mean ± S.D.	CV%
Within-run				
cells	1.07 0.11	10.5	0.13 ± 0.03	23.5
plasma	7.79 ± 0.78	9.9	1.05 ± 0.10	9.9
Between-run				
cells	1.05 ± 0.26	2.5	0.17 ± 0.05	31.3
plasma	7.72 ± 0.26	3.4	1.00 ± 0.04	4.1

each sample five times for a total of 45 cellular and plasma results.

Table 21 shows the results of precision studies done on both the red blood cells and the plasma . The mean, standard deviation, and coefficient of variation were calculated based on the running of one sample four times.

Analytical recovery studies were also performed. These were accomplished by adding 2.5 µg/mL of the corresponding isomer of interest to either the cells or the plasma and analyzing the sample as normally performed. The results of such recovery studies are shown in Table 22.

Representative chromatograms, as derived by the IBM 9000, are shown in Figures 14–22 . Figures 14–16 show a 5.0 µg/mL standard of alpha–, beta–, and gamma–tocopherol, respectively, Figure 17 shows a 4.0 µg/mL standard of delta–tocopherol. A chromatogram of the alpha–gamma standard used in the External Calibration Table is shown in Figure 18 . Reconstructed chromatograms of representative normal individual and sickle cell patients' cells and plasma are shown in Figures 19–22 .

TABLE 21

PRECISION OF TOCOPHEROL ANALYSIS (n=4)*
(µg/mL)

Cells	Alpha	Gamma
	1.42	0.25
	1.31	0.22
	1.48	0.26
	1.41	0.22
Mean	1.41	0.26
S.D.	0.07	0.05
C.V.	5%	19%
Plasma	11.17	2.27
	11.17	2.21
	10.95	1.89
	9.77	1.89
Mean	10.77	2.07
S.D.	0.67	0.21
C.V.	6%	9.9%

n=number of samples

TABLE 22

ANALYTICAL RECOVERY OF ALPHA- AND GAMMA-TOCOPHEROL ADDED TO PLASMA AND RED CELLS

	Alpha-tocopherol(µg/mL)			Gamma-tocopherol(µg/mL)		
	Added	Found	%Recovery	Added	Found	%Recovery
#1(P)*	0	13.32	------	0	2.34	------
	2.5	14.75	93	2.5	4.37	90
#2(P)*	0	10.77	------	0	2.07	------
	2.5	12.31	93	2.5	4.10	86
#3(C)*	0	1.39	------	0	0.27	------
	2.5	3.31	85	2.5	1.71	62
#4(C)*	0	1.29	------	0	0.23	------
	2.5	3.31	87	2.5	1.71	63

* P = plasma; C = cell

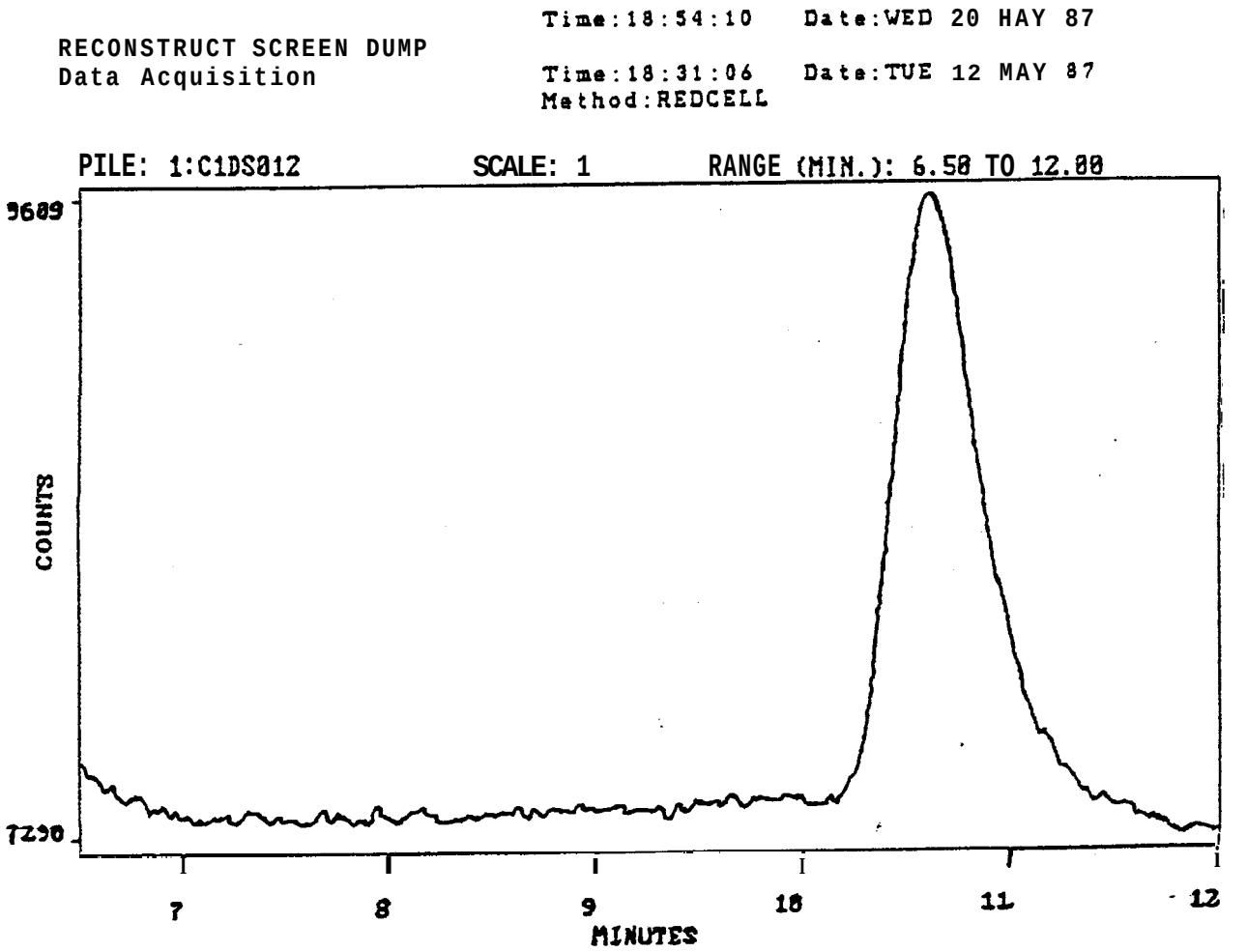


Figure 14. Chromatogram of 5.0 µg/mL of Alpha-tocopherol

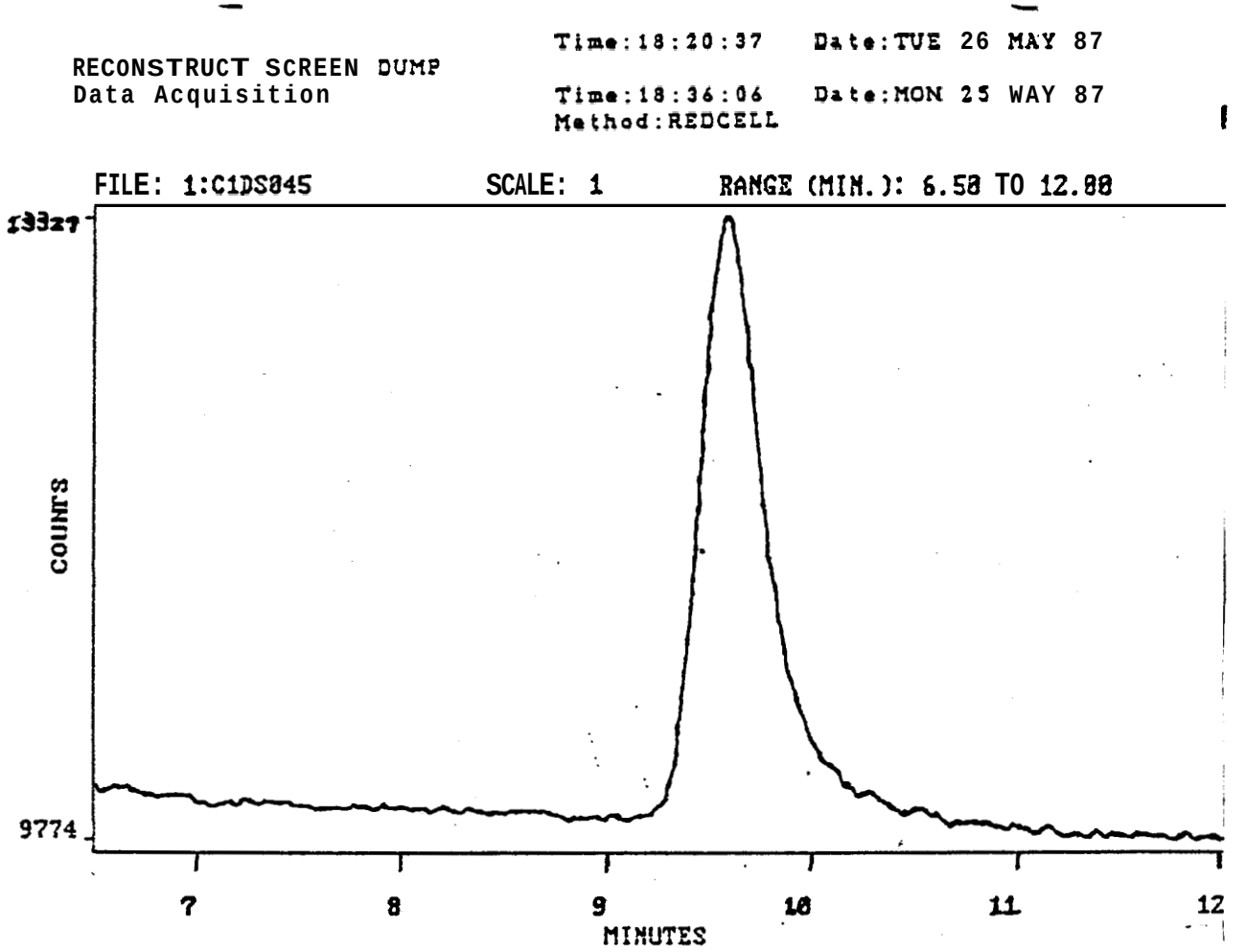


Figure 15. Chromatogram of 5.0 μg/mL Beta-tocopherol

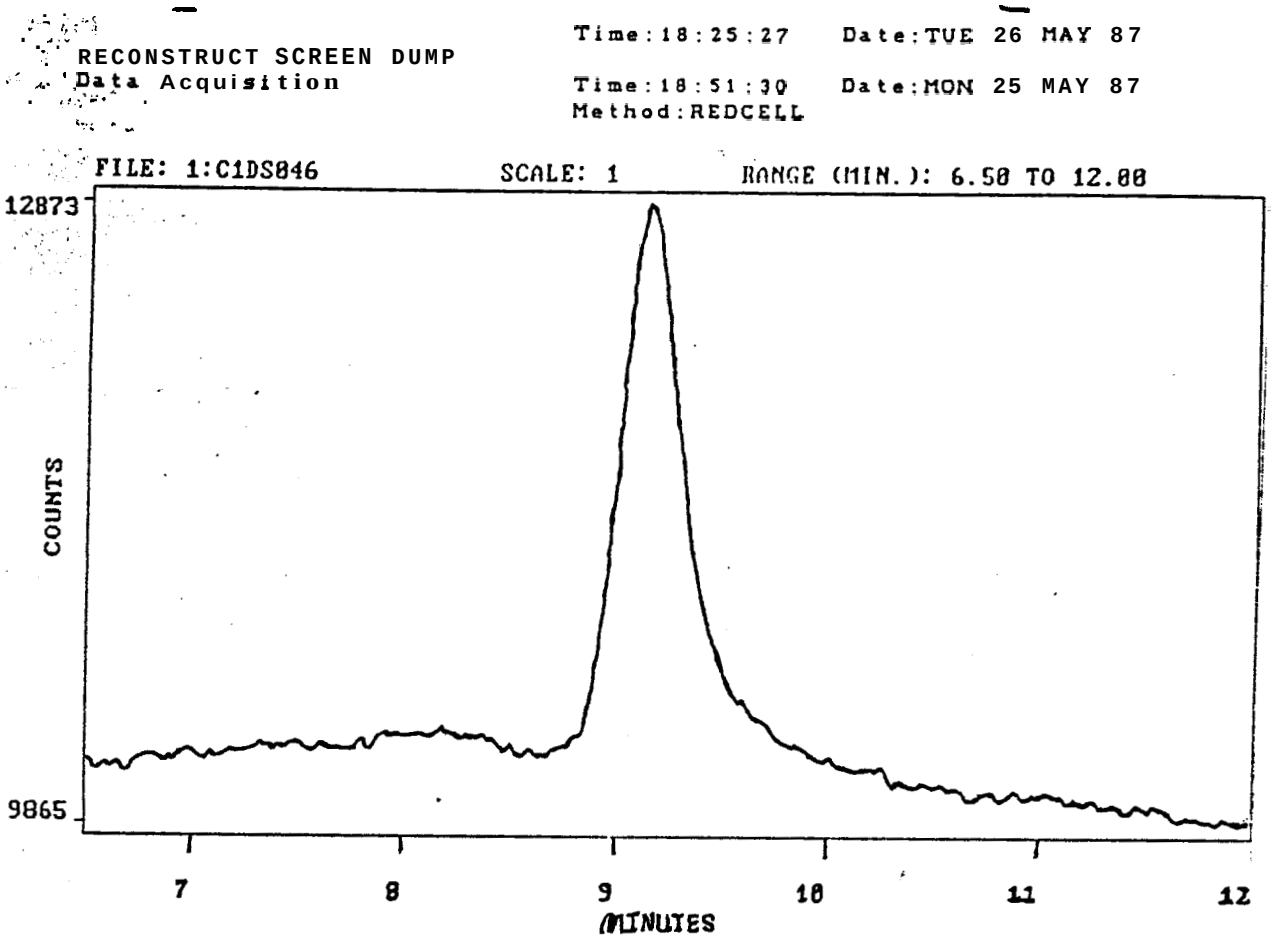


Figure 16. Chromatogram of 5.0 µg/mL Gamma-tocopherol

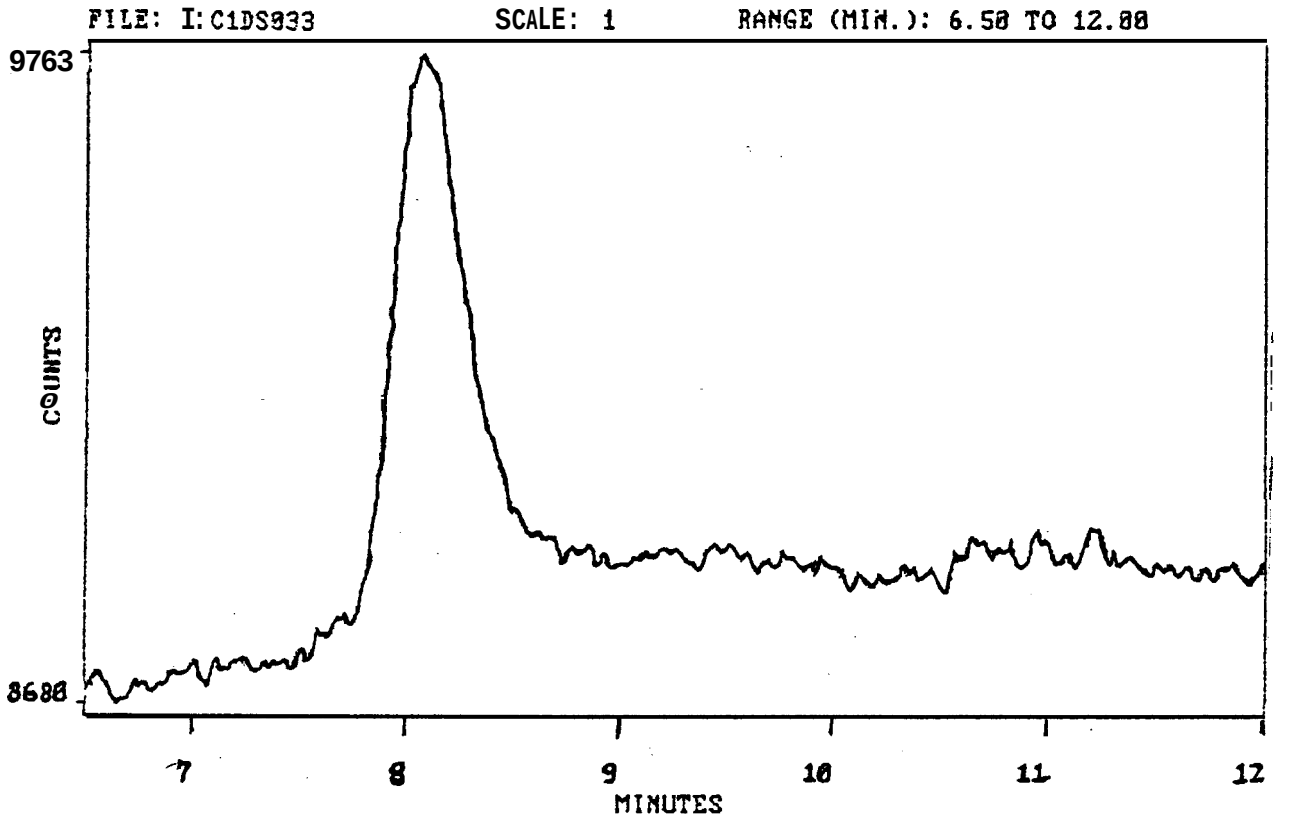


Figure 17. Chromatogram of 4.0 μg/mL Delta-tocopherol

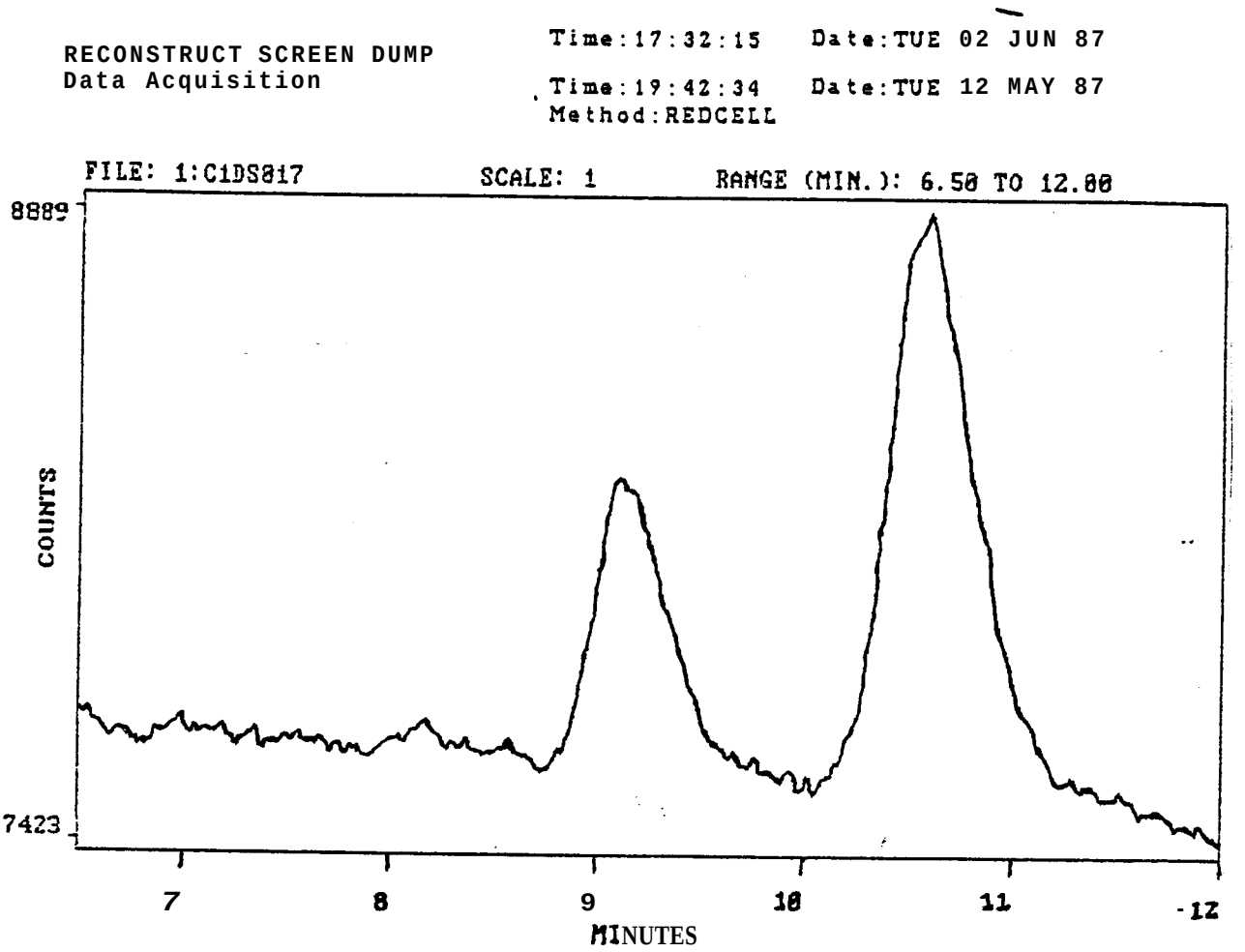


Figure 18. Chromatogram of Standard Mixture in External Standard (0.625 μg/mL gamma-tocopherol and 2.5 μg/mL alpha-tocopherol)

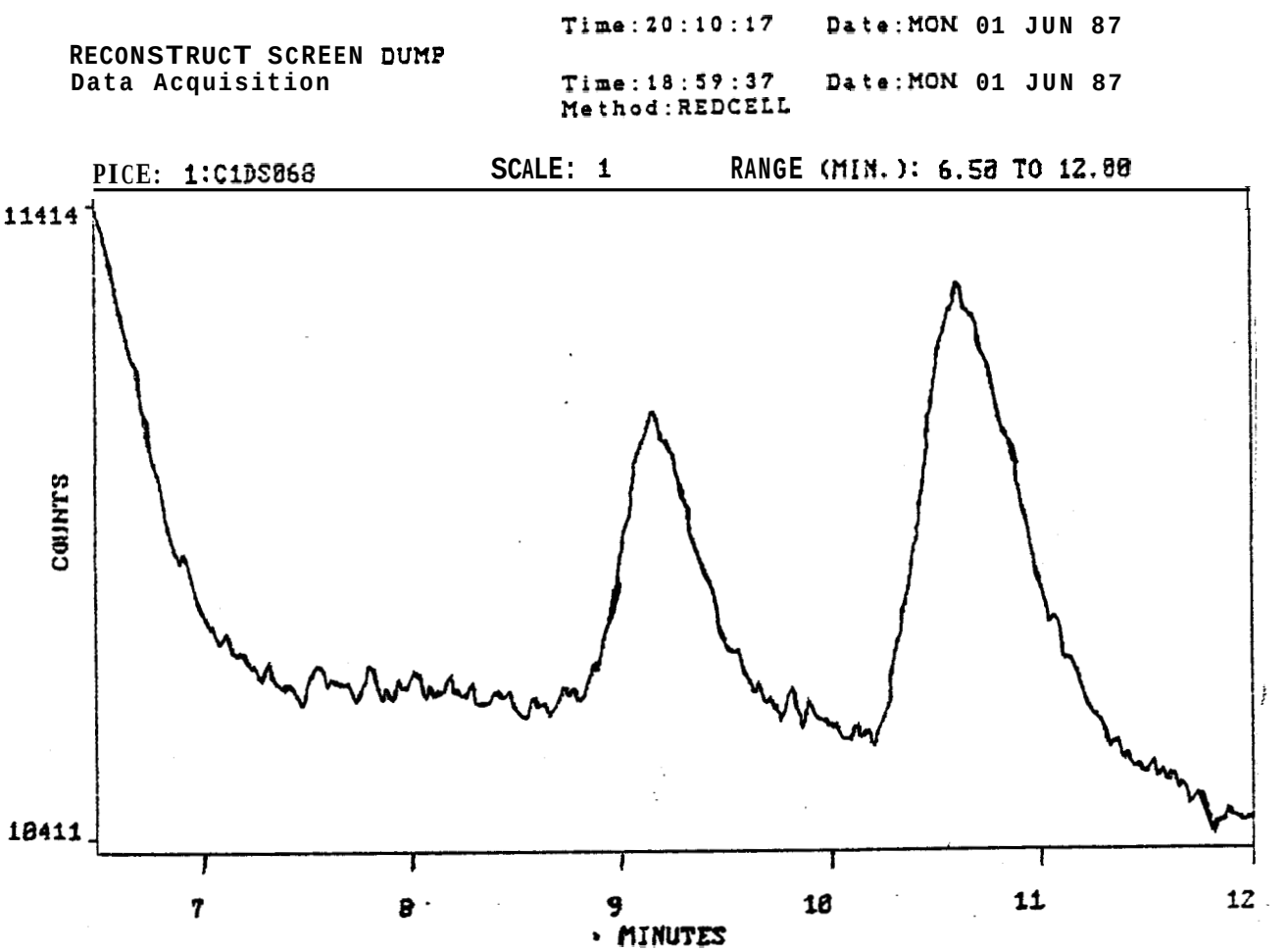


Figure 19. Chromatogram of Vitamin E Isomers from a Normal Patients' Red Blood Cells

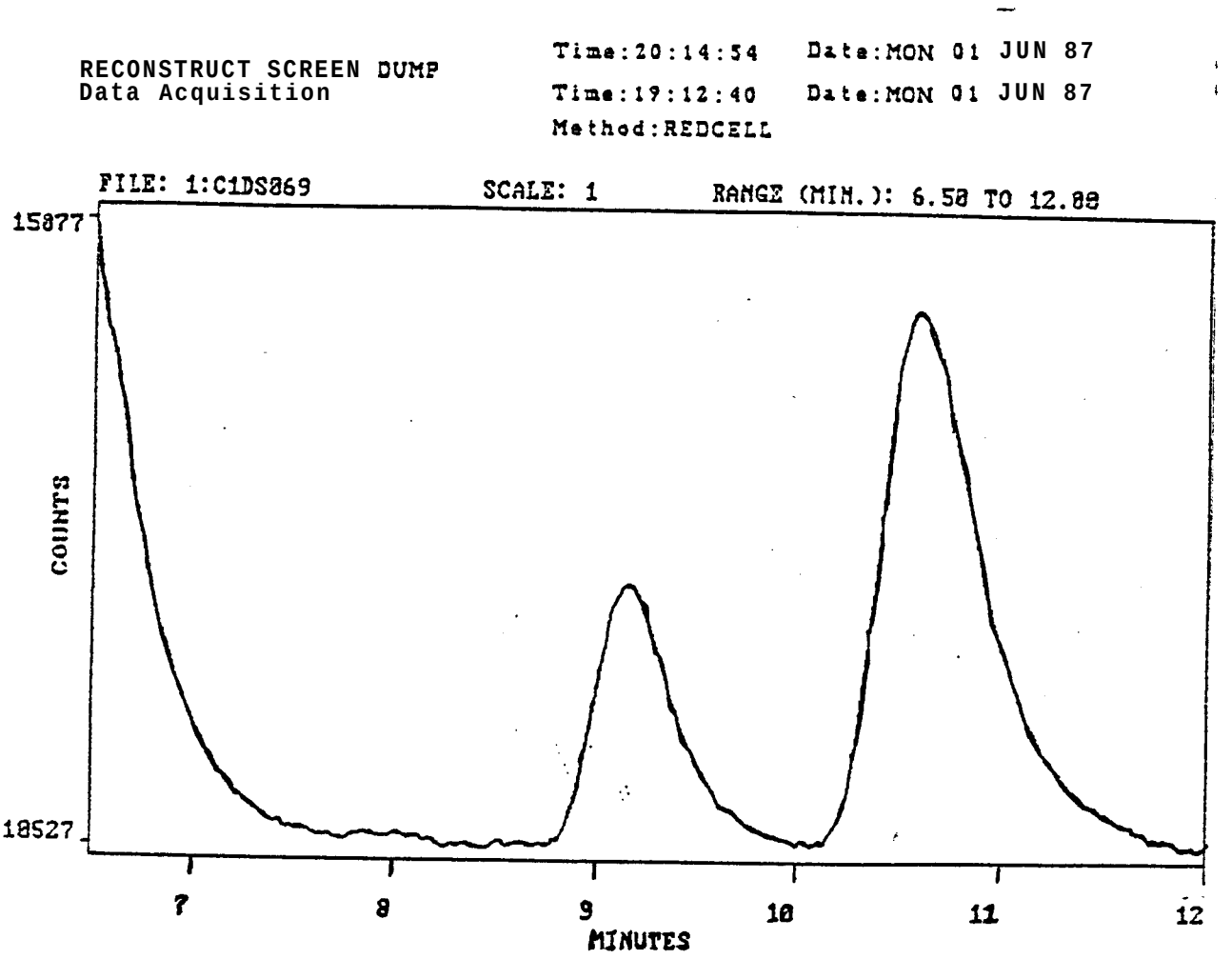


Figure 20. Chromatogram of Vitamin E Isomers from a Normal Patients' Plasma

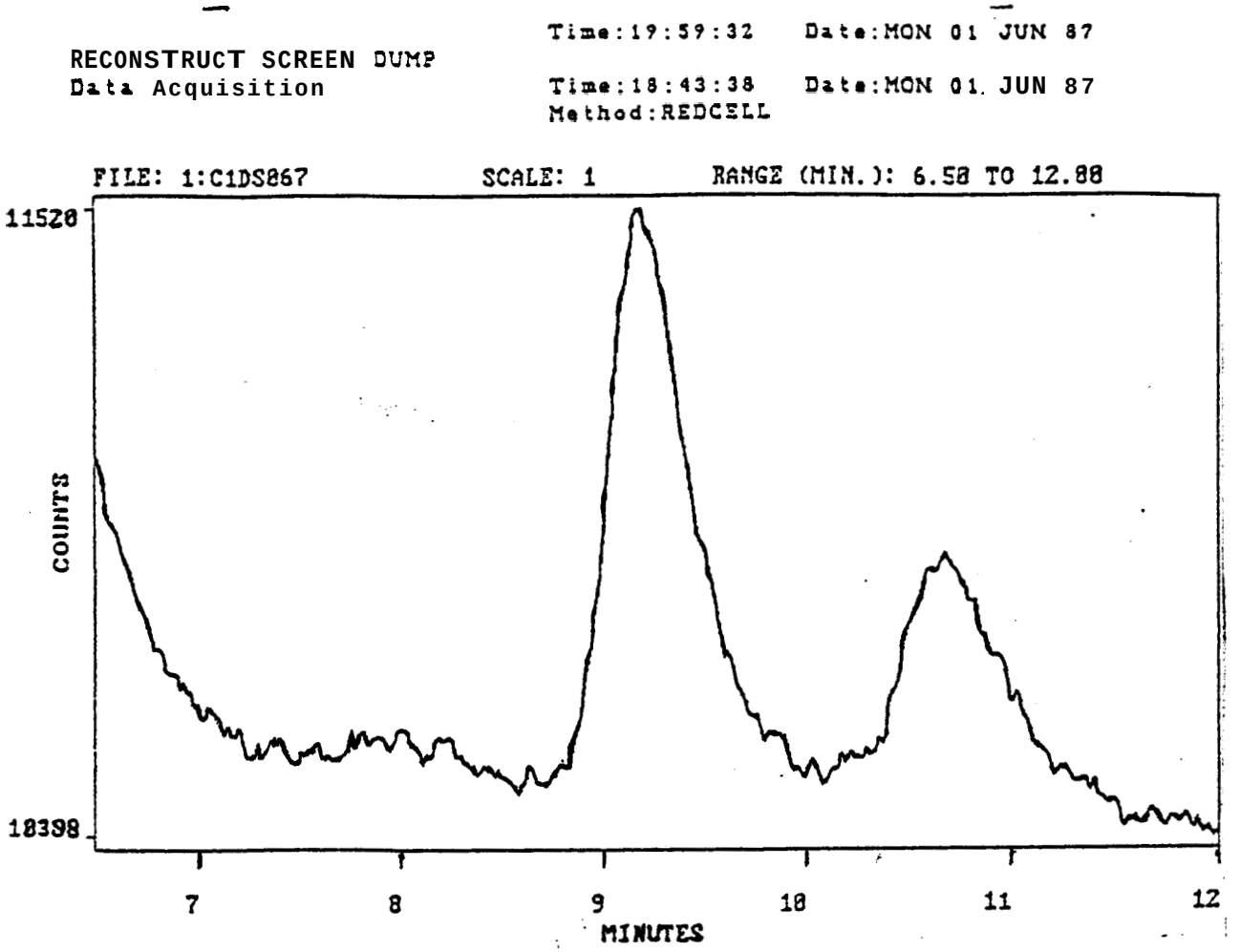


Figure 21. Chromatogram of Vitamin E Isomers from a Sickle Cell Patients' Red Blood Cells

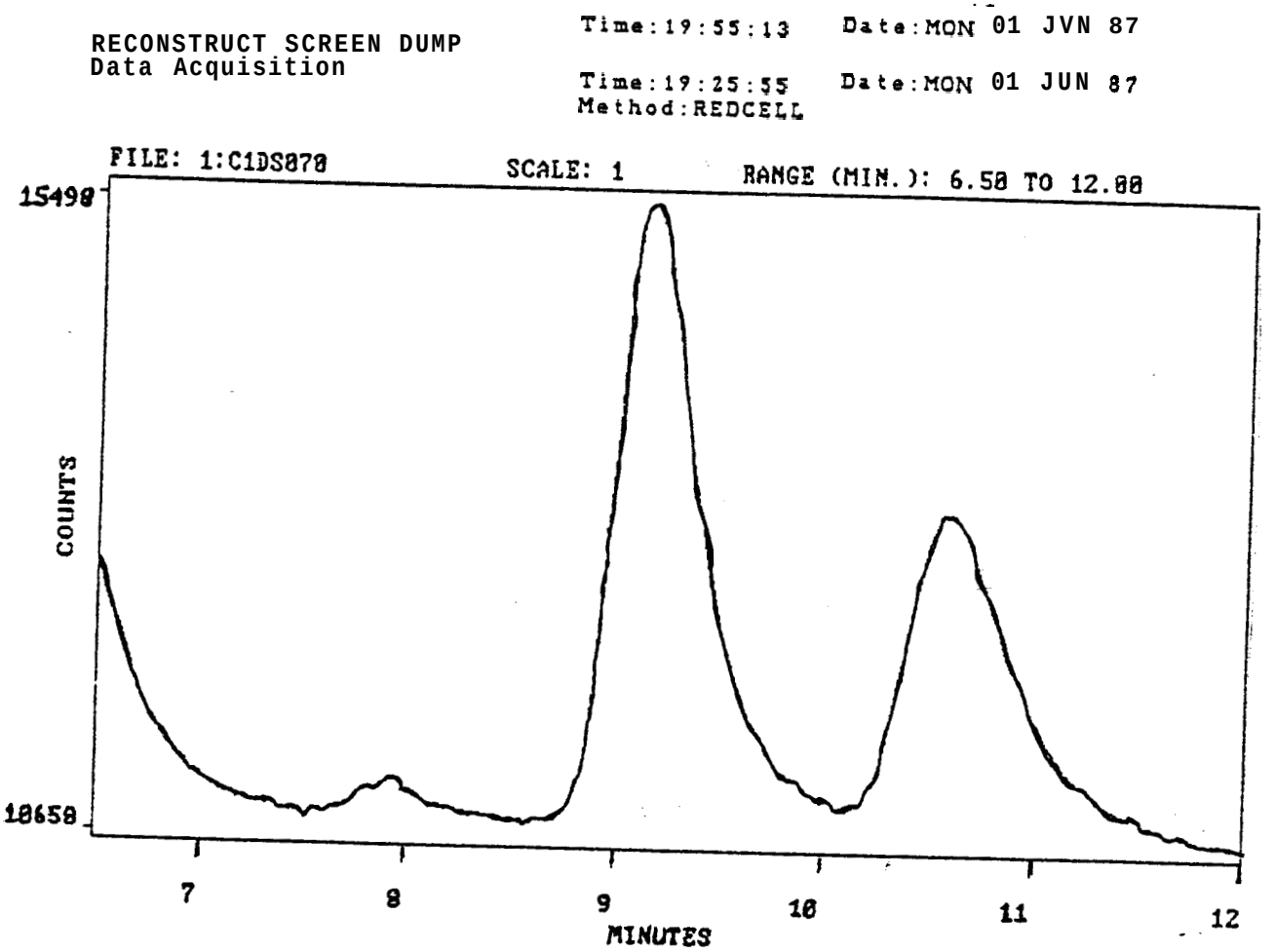


Figure 22. Chromatogram of Vitamin E Isomers from a Sickle Cell Patients' Plasma

Chapter VI

Conclusions

The results of this research have shown that HPLC can be utilized as a simple and sensitive technique for studying tocopherol levels in human erythrocytes and plasma. The HPLC method is capable of separating each of the four isomeric components of the incubation mixture as alpha, beta and gamma, and delta. Evaluation of column performance showed typical behavior for a reversed-phase column. The number of theoretical plates (10,816) agreed well with the manufacturer's expected values (30) and exemplifies the high degree of efficiency achievable with present day analytical columns (24).

Definitive identification of the alpha and beta-gamma peaks was shown consistently by reproducible peaks at 10.4 and 9.4 minutes, respectively. Further work is needed to prove whether the peak eluting at 8 minutes is delta-tocopherol or perhaps extraneous fluorescence from the method. The quantity of delta anticipated to be found is so low that variability in its detection could be due to using the same sensitivity selection as was used for the detection of the alpha-and beta-gamma isomers. A potential solution is to analyze for delta- at a different sensitivity setting than that utilized for the other isomers. Perhaps this would lead to better detection of this isomeric form. Another possible solution would be to divide the 2.0 mL hexane extract, evaporate a 1.0 mL portion to dryness and reconstitute this to a smaller volume in order to concentrate the delta isomer.

This could then allow simultaneous analysis of the aforementioned isaners by utilization of the same sensitivity setting.

The standard curves for each of the isomeric forms show linearity over a wide range of concentration, but more importantly exhibited linearity over the range found in normal human red cells and plasma (32). This allows the investigator to use a single external standard to monitor separate sample analysis runs. Consequently, acceptance of the external standard result allowed acceptance of patient sample results. This created a high degree of confidence in running and eliminated concern introduced by the extensive sample pretreatment. Further confidence in this method was generated as a result of reproducibility, precision and recovery studies for the isomers in most instances.

Reproducibility studies (Table 18) showed coefficients of variation of 10% or less for within-run and between-run studies performed on the alpha isomers in red cells and plasma. The gamma isomer showed the same coefficent of variation for the plasma measurements, but much higher (23.5 and 31.3%, respectively) for the red cell within-run and between-run results. It is speculated that this increased coefficient of variation is due to the much smaller quantity of gamma present in cells than in plasma (approximately a 10-fold difference in concentration) rather than an actual method problem. This coefficent of variation could possibly be lowered by concentrating the hexane extract by evaporation and reconstitution, thus allowing for an increase in the gamma component. Overall, however, the reproducibility

studies indicate that this method is acceptable in comparision with others currently in use (33,34).

Precision studies (Table 19) indicated similar results for coefficients of variation as were achieved with the reproducibility studies. The alpha component displayed coefficients of variation of 5 and 6%, respectively for cells and plasma, whereas, gamma showed 19% for cells and 9.9% for plasma. Again, the concentration of the latter isomer is considerably lower than its alpha counterpart and concentration of the hexane extract could possibly remedy the larger coefficients of variation.

Analytical recovery of alpha and gamma tocopherol in both plasma and cells were also evaluated. Table 22 shows that alpha's recovery in both cells and plasma was at least 85% or more. Gamma's recovery was the same percentage for plasma recoveries, but cellular values were much lower at approximately 62%. Once more, the cellular level of gamma is low in comparision to the plasma gamma level. Apparently, such a decrease affects the appearance of the cellular recovery of this isomer. However, the plasma recovery of this isomer indicates that the method is capable of good recovery when the level of the gamma component is higher.

The mean tocopherol levels for normal patients' red cells and plasma (Table 11) agree well with those of published results (17, 20, 27, 35, 36 and 37). Data utilized in these calculations were selected or rejected on the basis of the 2.5d rule (38). This rule is applied as follows:

1.) Compute the mean and the average deviation of the 'good results'.

2.) Find the deviation of the suspected result from the mean of the 'good' ones.

3.) If the deviation of the suspected result from the mean of the 'good' ones is at least 2.5 times the average deviation of the 'good' results, then reject the suspected result. Otherwise, retain it.

The mean plasma tocopherol levels of each isomer are approximately ten times the concentration of the corresponding cellular isomer in normal patients. The mean cellular alpha concentration of Sickle Cell patients is approximately the same as that for the normal patients. However, the gamma level in these patients is nearly three times that of a normal patients' red cell level. This resulted in an overall greater total tocopherol level in the red blood cells of Sickle Cell patients than in the RBC's of normal patients.

Comparision of plasma alpha and gamma between normal and Sickle patients showed slightly higher gamma levels in the Sickle patient, and-alpha levels approximately one-half those of normal patients. Total tocopherol in plasma was slighlty higher in normal patients. In summary, Sickle Cell patients had higher levels of red cell and plasma gamma tocopherol, but lower levels of plasma alpha tocopherol when compared to normal patients.

Based upon the description of correlation matrices given in the previous chapter, numerous relationships can be seen in Tables 14-17. Of these, the more notable follow:

(1.) A strong negative correlation exists between the alpha in red cells and alpha in plasma of Sickle Cell patients (-.8170).

(2.) A good positive correlation exists between alpha in the red cells of normal patients versus alpha in Sickle Cell patients (.8038).

(3.) A strong negative correlation is seen between gamma in normal cells and gamma in Sickle Cell patients (.-7060).

(4.) Another strong negative correlation is seen between gamma in normal plasma and gamma in Sickle Cell patients plasma (-.6869).

(5.) A positive correlation (.6869) exists between alpha in normal and gamma in Sickle Cell patients plasma.

Table 12 shows the 90, 95, and 99% confidence intervals on the mean data as listed. Such values give the reader an indication of the probability of an individual observation falling outside of these limits (38). A comparision of these indicates that normal patients' and Sickle Cell patients' red cell alpha values assume approximately the same range as do total plasma levels for both populations.

The ratio of alpha to gamma in both cells and plasma of normal patients (0.21 and 0.22) was nearly one half the ratio found in Sickle Cell patients (0.51 and 0.56). Therefore, normal patients show a much higher concentration of alpha in comparision to gamma and Sickle Cell patients show nearly equal concentrations of both isomers. The ratio of alpha to gamma for normal patients agrees well with the published ratio of 0.27 (39).

Raw data for normal patients was evaluated and two samples were rejected based upon the 2.5d rule (38). A recalculation of the mean, standard deviation, and standard error is shown in Table 11. As can be seen, rejection of these two values resulted in a much smaller standard deviation for the alpha and total plasma values.

The Student's-t Test (38,40) was utilized to determine if any significant differences existed between normal and Sickle Cell disease patients red cell and plasma values. A summary of these calculations is shown in Table 23. A significant difference was observed between the gamma and total red cell levels up to the 99.5% level. Plasma alpha levels (all data) were significantly different up to the 99% level, whereas, the plasma gamma was significant only to the 95% level. The Student's-t values presented were evaluated with the degrees of freedom shown in Table 23. In summary, statistically significant differences between normal and Sickle Cell patients were observed for gamma within the red cell and alpha within the plasma.

Suggestions for further research on Vitamin E levels in the red cells and-plasma of normal and Sickle Cell patients include the following:

(1.) the use of heparinired samples, which are believed to result in increased ratios of RBC:plasma tocopherol (41)

(2.) investigation of plasma lipid levels simultaneously with tocopherol in order to see if the transportation of tncopherol from plasma to RBC membranes changes when the lipid concentration increases (16)

TABLE 23

SUMMARY OF STUDENT-t TEST RESULTS

Number of Observations (N)	degrees of freedom (N-1)	95%	97.5%	99%	99.5%
17	16	1.75	2.12	2.58	2.92
15	14	1.76	2.14	2.62	2.98

Patient Results

cells (n=17)		plasma (n=15)	
Alpha	1.09	Alpha(all data)	2.67*
Gamma	3.38**	Total	3.44**
Total	1.11	Alpha	8.01***

*Statistically significant difference up to the 99.5% level.
**Statistically significant difference at all levels.
***Data reanalyzed based on criteria for rejection with the use of the 2.5d rule. Statistically significant at all levels.

(3.) an understanding of why alpha-tocopherol is the major tocopherol in both red cells and plasma when a typical American diet provides two to four times as much gamma-tocopherol as alpha (42)

(4.) what the levels of the isomeric forms of tocopherol are in patients heterozygous for Sickle Cell anemia are in comparision to those of homozygous individuals

(5.) what the levels of the isomeric forms of tocopherol are in patients with other hemoglobinopathies, such as Beta-thalassemia (43) and

(6.) is the tocopherol level in Sickle Cell disease patients the same for those in remission as it is for those in crises, and is the difference seen in this research between normal patients and Sickle Cell disease patients the result of analyzing these patients in crises?

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