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SEPARATION OF ISOMERS AND STRUCTURALLY RELATED COMPOUNDS USING CYCLODEXTRINS AS MOBILE PHASE AND BUFFER ADDITIVES IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS.

By

Brian Spencer

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree Doctor of Philosophy

Department of Chemistry
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March 1996

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to Marie Claire
and my parents, Roman & Diana

SEPARATION OF ISOMERS AND STRUCTURALLY RELATED COMPOUNDS USING CYCLODEXTRINS AS MOBILE PHASE AND BUFFER ADDITIVES IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS.

ABSTRACT

Derivatized β-cyclodextrin was used as mobile phase additive for the high performance liquid chromatographic resolution of amino acid derivative enantiomers while using an achiral C_{18} stationary phase. Dimethyl-β-cyclodextrin gave improved enantioselectivity over the underivatized β-cyclodextrin. The effects of cyclodextrin concentration, pH, and methanol and buffer concentration on enantioselectivity and capacity factor were examined.

β-Cyclodextrin and two derivatized cyclodextrins were utilized as mobile phase additives for the liquid chromatographic resolution of equilin and estrone as well as estrone from 2-, 4-, and 16α-hydroxyestrone. β-cyclodextrin proved to be suitable in these separations but the modified β-cyclodextrins provided better resolution. Apparent inclusion complex strengths were calculated for estrone and its derivatives were calculated for each cyclodextrin.

Micellar electrokinetic chromatography using sodium dodecyl sulphate with β-cyclodextrins was found to give comparable or improved separation of a series of fat-soluble vitamins over reversed-phase high performance liquid chromatographic techniques with cyclodextrins in the mobile phase.

Charged carboxymethyl-β-cyclodextrin was used in the capillary electrophoretic separation of a series of nine tricyclic antidepressants. Cyclodextrin alone was successful in separating some of the compounds under investigation while complete separation required the addition of a micellar pseudophase. A variety of coatings were investigated to reduce or eliminate the electroosmotic flow.

SÉPARATION DES ISOMÈRES ET DES MOLÉCULES DE STRUCTURES SIMILAIRES AVEC LES CYCLODEXTRINES COMME ADDITIFS EN PHASE MOBILES ET EN TAMPONS AVEC LA CHROMATOGRAPHIE LIQUIDE À HAUTE PERFORMANCE ET L'ÉLECTROPHORÈSE CAPILLAIRE

RÉSUMÉ

Un dérivé de la β-cyclodextrine a été utilisée comme additif de phase mobile pour la résolution par chromatographie liquide des énantiomorphes d'acides aminés dérivés en utilisant une phase stationaire C_{18}. Le diméthyle-β-cyclodextrine a produit un amélioration en énantiosélectivité par rapport à la cyclodextrine non-modifiée. Les effets du pH et des concentrations de la cyclodextrine, du méthanol, et du tampon KH_2PO_4 sur l'énantiosélectivité et la rétention ont été examinés.

La β-cyclodextrine et deux derivées ont été utilisées comme additif de phase mobile pour la séparation chromatographique d'équiline et estrone et d'estrone de 2-, 4-, and 16α-hydroxyestrone. Les β-cyclodextrines modifiées ont produit une meilleure séparation que la β-cyclodextrine non-modifiée. Les constantes de formation ont été calculées pour l'estrone et ses dérivés pour chaque cyclodextrine étudiée.

La chromatographie électrocinétique micellaire, en utilisant du dodécyl sulfate de sodium avec de la β-cyclodextrine dans le tampon, a donné un séparation comparable ou supérieure à celle de la chromatographie liquide pour la séparation d'une série de vitamines liposolubles.

Une cyclodextrine ionique a été utilisée dans la séparation électrophorique en capillaire d'une série d'antidépressants tricycliques. La cyclodextrine employée seule a pu séparer quelques unes des molécules tandis que l'addition d'une phase micellaire les ont toutes séparées. Un variété d'enduits ont été étudiés dans le but de réduire ou d'éliminer le courant électroosmotique.

ACKNOWLEDGEMENTS

I would like to express many thanks to Professor William C. Purdy for his support and guidance throughout my stay at McGill. The opportunity to pursue my research interests in an environment of independence was greatly appreciated.

I would also like to express my gratitude to some of the people who made my stay at McGill a pleasant one. Song Li for getting me started on my project; Tanya Tadey, Guy Légère, Jeff Chance, and Melodie Schweitzer for their friendship, support, and countless discussions; and special thanks to my friend Wenbin Zhang for helping to steer me on the right course. I'd also like to thank him for helping me get started with capillary electrophoresis.

I'd also like to express my gratitude to my family. First to my parents for their encouragement and support while pursuing my educational ambitions to their highest level. I'd also like to thank my wife, Marie Claire, for her love and understanding, and for putting up with me while I was writing my thesis.

Finally, I would like to thank the Department of Chemistry at McGill University for demonstrator assistantships, the Natural Science and Engineering Research Council of Canada (NSERC) for financial support, and the Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (FCAR) for my scholarships.

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GLOSSARY

α	separation factor
ζ	zeta potential
μ_{ep}	electrophoretic mobility
16α-HE	16α-hydroxyestrone
2-HE	2-hydroxyestrone
4-HE	4-hydroxyestrone
C_{18}	octadecylsilane stationary phase
CD	cyclodextrin
CE	capillary electrophoresis
CM-β-CD	carboxymethyl-β-cyclodextrin
CZE	capillary zone electrophoresis
DM-β-CD	dimethyl-β-cyclodextrin
DNB	dinitrobenzoyl
DNP	dinitrophenyl
EOF	electroosmotic flow
HE-β-cd	hydroxyethyl-β-cyclodextrin
HPLC	high performance liquid chromatography
k'	capacity factor
LC	liquid chromatography
L_d	length of capillary to detector
L_t	total length of a capillary
MEKC	micellar electrokinetic chromatography
R_s	resolution
SDS	sodium dodecyl sulphate
t_m	migration time
t_o	mobile phase retention time
t_R	retention time
t_w	peak width
v_{ep}	electrophoretic velocity

CHAPTER 1

LIQUID CHROMATOGRAPHIC AND ELECTROPHORETIC METHODS OF ANALYSIS

1.1 INTRODUCTION

Analytical chemists are frequently presented with samples in which the analyte under consideration is part of a complex matrix. The challenge is to identify and quantify that analyte without interference from the matrix. The most common approach is to isolate that compound from its fellow matrix components using a variety of separation techniques. There are many techniques in the field of the separation sciences; this chapter will provide an overview of the two techniques utilized for the work in this thesis, high performance liquid chromatography (HPLC) and capillary electrophoresis (CE). HPLC is well established as one of the premiere tools used by analytical chemists today yet its usefulness has not been completely exploited and the technique is still the subject of much research. Modern CE in fused silica tubes was first reported in 1981 by Jorgenson and Lukacs [1]. It has since become the subject of an impressive amount of research and consequently CE has become a powerful separation method, both by itself and as a complementary technique to HPLC.

1.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The term "chromatography" refers to a separation process in which the components in a mixture are separated from each other by distributing themselves between two distinct phases, a stationary phase and a mobile phase [2]. In liquid chromatography (LC), the stationary phase is made up of solid particles and the mobile phase is in the liquid state. Liquid chromatography is used extensively for the analysis, purification, and preparation of chemical compounds. The traditional forms of liquid chromatography include adsorption, partition, thin-layer, paper, and ion-exchange. Modern LC is referred to as high performance liquid chromatography (HPLC) and features improvements in instrumentation which allow for better separations [3].

1.2.1 The Chromatographic Process

Figure 1.1 is a schematic of a chromatographic separation for a two component system migrating through a column. Two processes are obvious from this diagram. The first is *differential migration*. Each component is moving through the column at different rates. The relative migration rates dictate whether two components will be well separated. The greater the difference in migration rates, the better the separation. The difference in rates results from the equilibrium distribution of a particular solute between the stationary and mobile phases. A solute that favours the stationary phase more than another solute will be retained longer. The degree of partitioning between the stationary and mobile phases is determined by experimental parameters, such as the composition of the stationary and mobile phases, flow rate, and temperature [3].

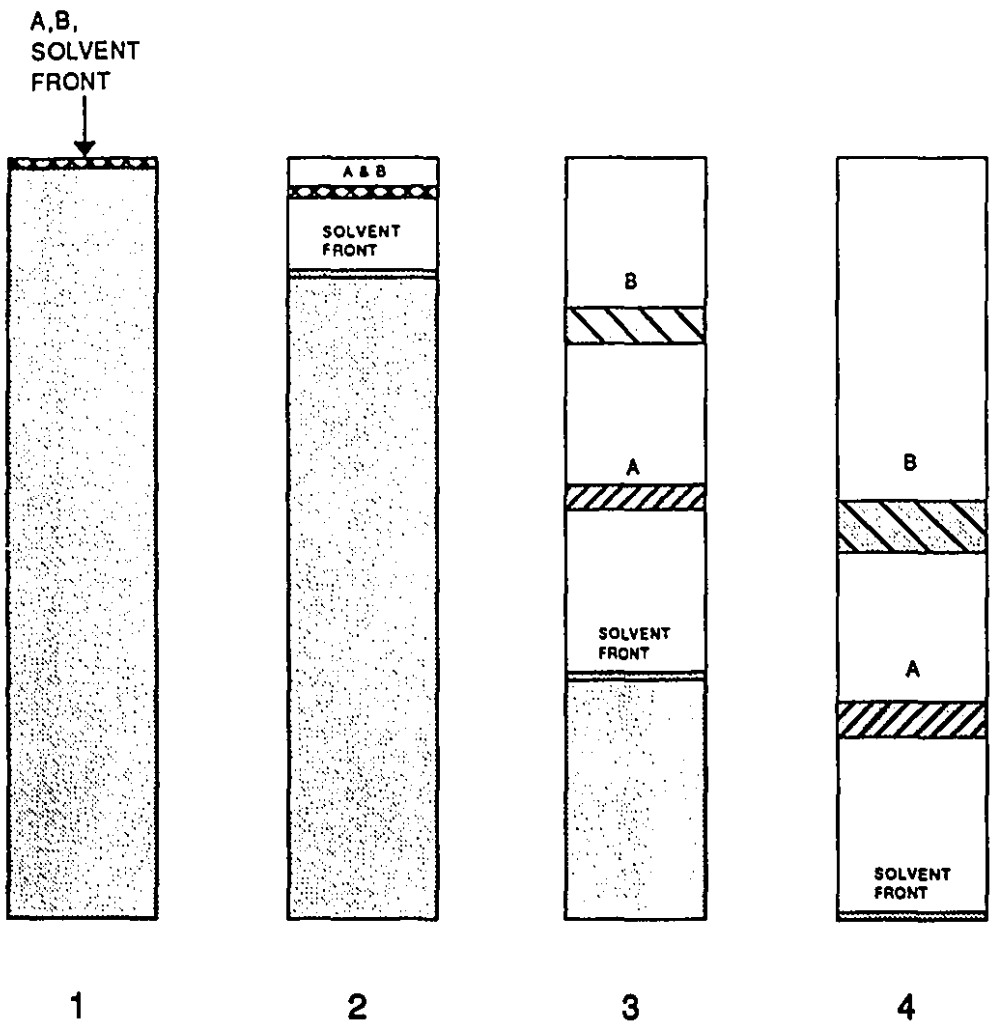

Figure 1.1 Hypothetical Chromatographic Separation of Two Components (adapted from [3])

The second process operating on the two components in Figure 1.1 is solute *band broadening*. The average migration rate of individual molecules within a given solute band is not identical so they tend to spread out over time. Band broadening has a detrimental effect on the separation of closely eluting bands since it causes the bands to overlap. Broadening is a result of physical or rate processes. Band broadening has 6 main sources [3]:

(i) Eddy diffusion: Broadening arises from different flow streams that the solvent follows between particles in a packed column.

(ii) Mobile phase mass transfer: This refers to the different flow rates within a single flow stream due to the relative distance of molecules from the column particles. Molecules close to particles tend to slow down with respect to molecules that are further away.

(iii) Stationary phase mass transfer: This refers to different flow rates as a function of how far the solute molecules have penetrated into the stationary phase. Deeper penetrating molecules will flow slower than those that have not penetrated as far.

(iv) Stagnant mobile phase mass transfer: This occurs when solute molecules are slowed when trapped within the pores of a particle and remain stagnant for a short period of time.

(v) Longitudinal diffusion in the column: This is the tendency for molecules in high concentration regions to travel to one of lower concentration. This is usually insignificant in LC except at very low flow rates.

(vi) Extracolumn broadening. This is caused by diffusion in the fittings and tubing outside the column. In a well designed system, its effects are minimal.

When the separated solute bands elute from the column, the resulting chromatogram can be characterized by:

(i) The shape of the band. Ideally, it is Gaussian.

(ii) Retention time (t_R). This is the time required for the solute to pass through the column. It is constant for a given set of experimental parameters.

(iii) Band width, which is given by t_W.

(iv) Differences in retention time between the bands.

Under ideal circumstance, a solute undergoing a separation process should elute as a sharp spike at the detector. In other words, the sample plug should stay intact in the column. This ideal case is never realized and so the solute appears as a band that can usually be described as a Gaussian distribution. Over the years, scientists have tried to explain why and how this band broadening occurs. There are dozens of theories reported in the literature which try to explain solute band broadening. Some of these authors have built on previous theories while others attempted to disprove earlier concepts and presented their own view of band broadening. The plate theory of chromatography, which will be summarized later, was suggested by Nobel Prize winners A.J.P. Martin and R.L.M. Synge [4]. Martin and Synge's work was followed by the rate theory of chromatography, which was developed by a group of Dutch chemical engineers led by J.J. van Deemter [5]. Their theory was based on equations derived by L. Lapidus and N.R. Amundson [6]. Other theories for band broadening include Marcel Golay's rate theory as applied to stationary phase-coated capillary columns [7] and J.C. Giddings' random walk theory, which gives a purely statistical perspective of band broadening [8].

1.2.2 Retention in Liquid Chromatography

The retention time for a given solute can be expressed by:

$$t_R = t_0(1+k') \qquad (1.1)$$

where t_0 is the retention time of an unretained solute (usually the solvent front) and k′ is the capacity factor. The capacity factor is an important parameter in chromatography and is defined as the number of moles of solute in the stationary phase over the number of moles in the mobile phase. The value for k′ can be obtained by rearranging equation 1.1:

$$k' = \frac{t_R - t_0}{t_0} \qquad (1.2)$$

This parameter is a measure of column efficiency for a particular solute and is constant for a specific set of operating conditions. A greater value of k′ signifies a longer retention time.

1.2.3 Efficiency and Resolution in Liquid Chromatography

A chromatographic column can be regarded as a series of plates with equilibration between the stationary and mobile phases taking place at each plate. The greater the number of plates, the greater the number of times a solute will partition between the two phases resulting in a longer retention time [4]. The number of theoretical plates, denoted N, is given by:

$$N = 16\left(\frac{t_R}{t_w}\right)^2 \qquad (1.3)$$

N is a measure of column efficiency and should be nearly constant for all solute bands at a given set of experimental parameters. Packed columns in HPLC have a typical N values in the range of 5000-15000 [9]. Another measure for

column efficiency is the height equivalent of a theoretical plate, denoted HETP or simply H. The HETP is related to column length (L) and N by:

$$H=\frac{L}{N} \qquad (1.4)$$

As the value of H decreases, the efficiency of the column increases. Generally, the HETP is small for columns with small particles and systems with low flow rates.

The HETP is related to the flow rate velocity by the van Deemter equation [3,5]:

$$H=Au^{1/3}+\frac{B}{u}+Cu \qquad (1.5)$$

where u is the mobile phase velocity. The three terms in the equation originate from the main factors that contribute to band broadening. The terms represent eddy diffusion (A term), longitudinal diffusion (B), and resistance to mass transfer (C). Note that the contributions from A and C increase with flow rate while that of longitudinal diffusion (B) decreases it contribution to the HETP. The modernized version of the van Deemter equation discounts eddy diffusion due to improved column preparation techniques that minimizes microchannels in the packing bed [10]. The modernized equation also differentiates between the contribution due to mass transfer in the mobile ($C_M u$) and stationary ($C_S u$) phases and is given by:

$$H=\frac{B}{u}+C_S u+C_M u \qquad (1.6)$$

Resolution (R_s) can be defined as the degree to which two chromatographic bands are separated. This is a quantitative measure of the

relative separation of two adjacent bands and is the difference in retention times over the average band width:

$$R_S = \frac{(t_2 - t_1)}{1/2\,(t_{w_1} + t_{w_2})} \qquad (1.7)$$

The resolution between two peaks can also be expressed as:

$$R_S = 1/4\,(\alpha - 1)\sqrt{N}\left(\frac{k'}{1+k'}\right) \qquad (1.8)$$

where α is the separation factor, defined as the ratio of the capacity factors of the two solutes (i.e. k_2/k_1). There is no separation when α is unity. Optimizing resolution involves manipulating one of the three terms of equation 1.8. The three terms are:

(i) Separation selectivity (α-1): This is increased by optimizing the value of the separation factor through changes in the stationary and/or mobile phases.

(ii) Separation efficiency ($\sqrt{N}$): Resolution is controlled by using this parameter by changing the number of theoretical plates. This can be done by using a longer column or using smaller particles in the column.

(iii) Solvent strength ($k'/(1+k')$): Changing the solvent strength changes the ability of the mobile phase to provide large or small values for the capacity factor, k'.

1.2.4 High Performance Liquid Chromatography Instrumentation

Figure 1.2 is a schematic of a modern HPLC instrument. The pump must be able to deliver the mobile phase, which has been degassed and filtered, at a constant and reproducible rate. The pump is usually reciprocating using dual pistons to deliver the solvent, although positive displacement pumps

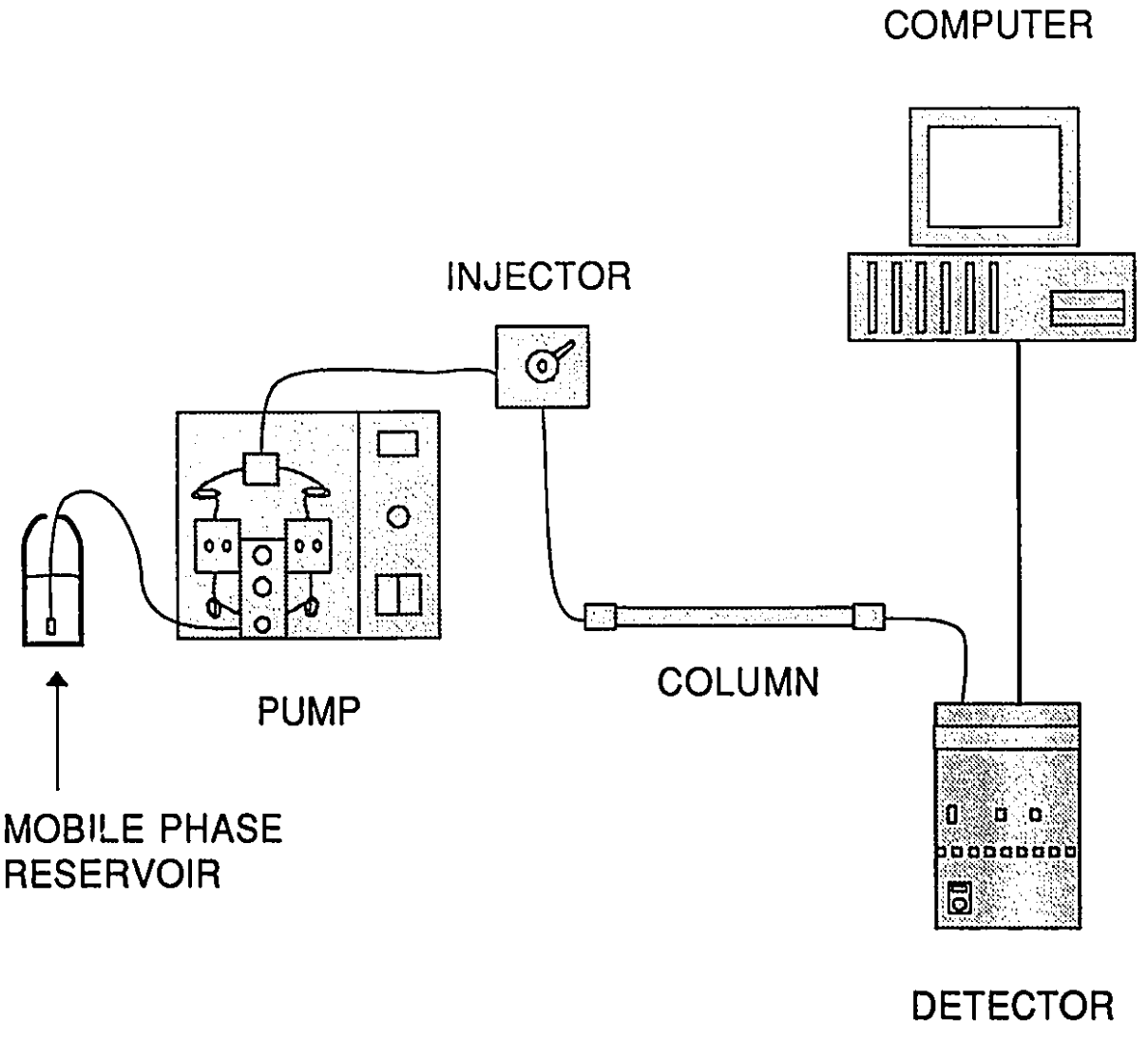

Figure 1.2 HPLC Instrumentation

have also been used. The mobile phase can be delivered isocratically (the mobile phase remains the same throughout the run) or in a gradient mode to optimize the k´ values of the individual bands.

The injector is usually an injection valve system or, more commonly with automation, an autosampler that is capable of running up to several hundred samples without the presence of an operator.

The detector must have high sensitivity, good selectivity, a large dynamic range, and respond independently of the mobile phase. Common detector types include UV/Visible and fluorescence spectroscopy, differential refractometry, electrochemical, and recently mass spectrometry. The latter is finding increased use as it is very sensitive and can provide qualitative molecular weight data about the sample. The computer is used for data acquisition.

The column is the heart of the chromatographic system as it is where the separations take place. The column is usually a 15-25 cm long stainless steel tube (inner diameter of 4.6 mm) containing 5-10 μm particles packed under high pressure. The majority of columns used today are organic stationary phases chemically bonded to the particles. They generally separate on the basis of the type and number of functional groups on the solute molecule. Examples of stationary phases range from non-polar C_{18} and C_8 to polar NH_2 columns. The use of silica as the inert support for the stationary phase provides for high column efficiency and stability. Preparation usually involves the reaction of surface silanol groups with the siloxane of the group to be bound.

High performance liquid chromatographic separations are run in one of two modes: normal-phase or reverse-phase [9]. In *normal-phase* chromatography, a polar bonded-phase column is used with a non-polar solvent, such as hexane or heptane. A small amount of a more polar solvent is sometimes

added. The solvent strength is varied by changing the concentration of the more polar component. The other mode of operation is *reverse-phase* chromatography, which uses a non-polar stationary phase with a polar mobile phase. Water is the principal solvent with miscible organic modifiers, such as methanol or acetonitrile, added to vary the solvent strength. Changing the selectivity in reverse-phase is more difficult than in normal-phase since water dominates the sample-stationary phase-mobile phase interactions [3]. One advantage of reverse-phase is that the pH can be varied to change the retention of ionized or ionizable groups. Salts can also be easily added to vary the selectivity. Reverse-phase is the more popular of the two modes due mainly to the development of bonded phases (such as the very popular octadecylsilane (ODS, or C18) column) and the preference of using aqueous mobile phases over non-polar organic mobile phases.

1.3 CAPILLARY ELECTROPHORESIS

Capillary electrophoresis achieves the same end as high performance liquid chromatography separation, yet does so via a completely different mechanism. In a capillary electrophoretic separation, separation occurs as a result in differences in migration through a liquid within a capillary in the presence of an applied electric field. CE developed from traditional electrophoresis, which is usually performed on a slab-gel. Slab-gel electrophoresis has become the premiere technique used for the separation of macromolecules in the biological sciences. The birth of modern capillary electrophoresis, which features high efficiencies and the ability to be automated, began in the mid-1970's [10-12] where separations took place using glass and teflon capillaries with internal diameters of 200-500 μm. CE was first performed with narrow bore fused silica capillaries in 1981 [1] and the field has since grown at an exponential rate. The large amount of interest in CE will broaden the applicability of electrophoretic separations in the future.

1.3.1 The Electrophoretic Process

When an electric field is applied to an electrophoretic medium, such as a slab-gel or capillary, a background electrolyte is necessary to carry the current. The background electrolyte is commonly known as the run buffer. Apart from allowing the passage of current, the buffer also maintains pH and, occasionally, provides solute interactions. The sample is introduced at one end of the electrophoretic medium and migrates towards the other end under the influence of the applied electric field. Traditional electrophoresis employs a detection staining process while CE uses an on-line detection scheme.

The main advantage of CE over conventional electrophoresis is the ability of the capillaries to dissipate the heat created by the resistance to the ionic current flowing between the two electrodes [14]. This increase in heat dissipation results from the high surface area-to-volume ratio one finds in narrow-bore capillaries. The much improved heat dissipation allows for the use of very high voltages during electrophoretic separations. Traditional electrophoresis, which is prone to excessive heating, is limited to an applied voltage of less than 1 kV. CE voltages are much higher with a practical upper limit of 30 kV. The increase in voltage leads to a much shorter migration time in CE over conventional electrophoresis, as the migration time is inversely proportional to the applied field:

$$t_m = \frac{L^2}{\mu_{ep} V} \qquad (1.9)$$

where L is the column length, μ_{ep} is the electrophoretic mobility and which will be defined later, and V is the applied voltage. A typical CE separation usually takes less than 30 minutes while conventional electrophoresis separations can take several hours for completion.

1.3.2 Capillary Electrophoresis Instrumentation

In capillary electrophoresis, the separation typically takes place in a fused silica tube filled with background electrolyte. Fused silica is used because of its durability and its ultraviolet transparency. The tube has an interior diameter of 25-100 μm and can be up to a meter long. A schematic of a typical CE instrument is shown in Figure 1.3:

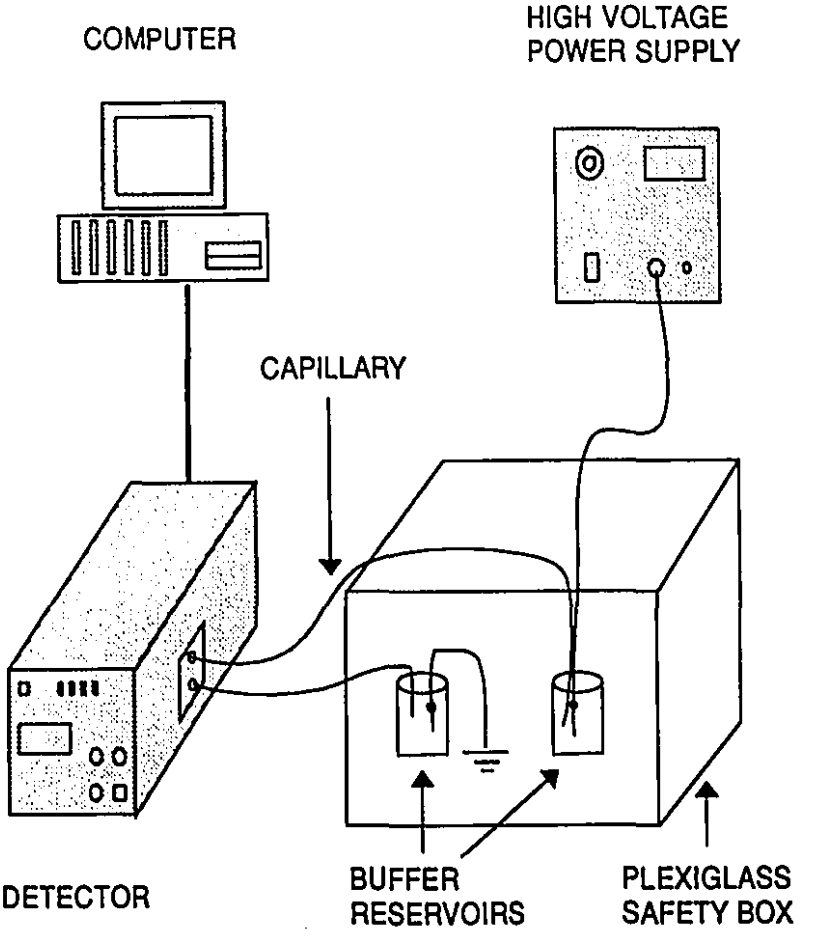

Figure 1.3 CE Instrumentation

Both ends of the capillary are placed into separate buffer reservoirs. One reservoir is connected to a high voltage power supply and the other to ground by platinum or graphite electrodes. The power supply delivers a potential of up to 30 kV. A small amount of sample, about 10 nL, is introduced at one end of the capillary.

Sample injection is usually electrokinetic or hydrodynamic. In electrokinetic injection, the sample end of the capillary and high voltage electrode are placed into the sample reservoir and the voltage is applied for a short period of time as the sample migrates into the capillary. The main disadvantage of this type of injection is that it introduces a bias on the basis of electrophoretic mobility. Hydrodynamic injection involves injection by pressure at the beginning of the capillary, suction at the end, or gravity flow where the sample vial with the capillary is raised above the other end of the capillary.

Upon application of a potential, typically 10 to 30 kV, the solutes in the sample migrate as a narrow band towards the other end of the capillary. In order for separation to occur, each solute must have different electrophoretic velocities in the capillary.

Detection in CE is usually on-capillary so as to not disrupt the flow of current. The most common detection type is UV where the polyimide coating on the capillary is burned off or removed with sulphuric acid. However, the small pathlengths offered by the narrow capillaries raise the detection limits significantly. The pathlength can be increased with the use of a Z cell [15]; however this requires the use of a specialized, more expensive capillary. Other types of detection used in CE include fluorescence, laser-induced fluorescence, and electrochemical detection, both potentiometric and amperometric. Another type of detection certain to find widespread use in the future is mass spectrometry. As in HPLC and gas chromatography, MS detection for CE is very sensitive and provides qualitative data about the sample.

1.3.3 Basic Concepts of Electrophoresis

The theoretical aspects of electrophoresis apply to both modern and traditional electrophoretic techniques. In the presence of an electric field, a charged particle will migrate with an electrophoretic velocity, v_{ep}, which can be described as:

$$v_{ep}=\mu_{ep}E \qquad (1.10)$$

where μ_{ep} is the particle's electrophoretic mobility and E is the applied potential [14]. The electrophoretic velocity can be determined experimentally from:

$$v_{ep}=\frac{L_d}{t_m} \qquad (1.11)$$

where L_d is the capillary length from injection point to the detector (separation distance) and t_m is the migration time to the detector.

The electrophoretic mobility is a function of its molecular weight, three-dimensional structure, and its degree of hydration in the supporting electrolyte. It is also a function of the electric double layer. The electric double layer is a result of the attraction of oppositely charged ions to a charged particle's surface. A diagram of the double layer can be seen in Figure 1.4. The double layer is comprised of two regions. The inner region is made up of compacted and adsorbed ions (called the Stern layer) while the outer layer (called the Gouy-Chapman layer) contains more diffuse ions that are distributed by electric forces (attraction and repulsion) and random thermal motion [16].

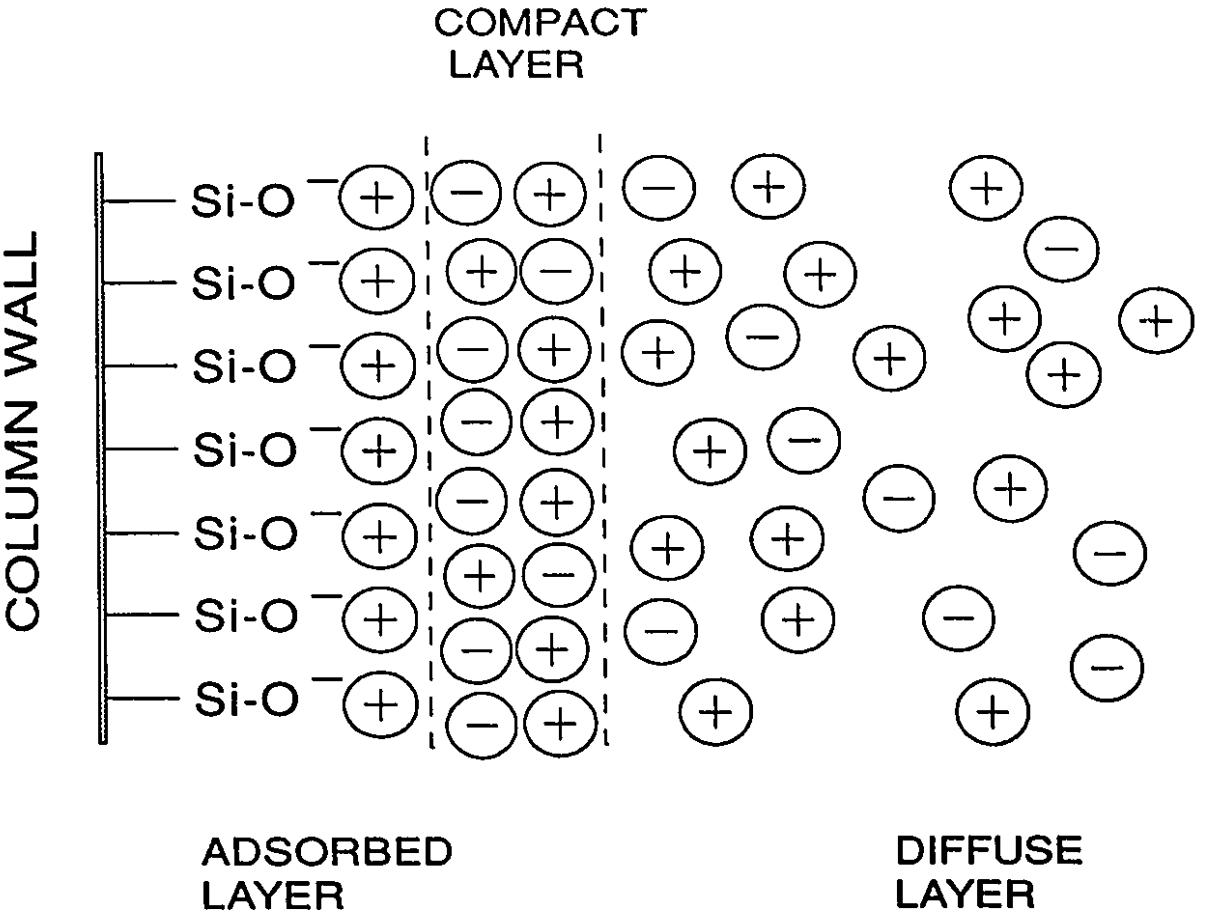

Figure 1.4 The Electric Double Layer (adapted from [16])

The consequence of this double layer is a potential across the two layers. This potential is called the zeta potential ζ and is given by equation 1.12.

$$\zeta = \frac{4\pi\eta\mu_{ep}}{\epsilon} \qquad (1.12)$$

η is the buffer viscosity and ε is the dielectric constant of the solution.

Rearranging equation 1.12 gives an expression for electrophoretic mobility:

$$\mu_{ep}=\frac{\zeta\epsilon}{4\pi\eta} \qquad (1.13)$$

Equation 1.13 shows that the electrophoretic mobility is a function of the zeta potential. Electrophoretic mobility, whether it be for individual particles or the electroosmotic flow, can be controlled by manipulating the zeta potential through changes in buffer ionic strength and pH. Thus, control of the zeta potential can be used to dictate the rate at which the sample will migrate through the capillary.

1.3.4 Electroosmosis

Electroosmosis is a very important parameter in capillary electrophoresis since almost all CE separations are performed with fused silica capillaries. Above a pH of about 3, the surface silanol groups (Si-OH) inside the capillary becomes ionized. The ionized silanol groups will attract cations in the buffer and form an electric double layer. In an applied field, the cations migrate towards the more negative electrode. The migrating cations drag the fluid in the buffer along with it. Due presumably to hydrogen bonding or van der Waals interactions, this flow is transmitted throughout the entire diameter of the capillary. This flow is referred to as the electroosmotic flow (EOF). The velocity of the electroosmotic flow is given by:

$$v_{eo}=\frac{e\zeta}{\eta}E \qquad (1.14)$$

The EOF can have a significant effect on the overall solute migration time. At low pH (<4), the silanol groups are partially or non-ionized so the EOF is insignificant. At higher pH (>6), where a lot of CE work is done, the EOF

increases significantly due to the high degree of ionization of the silanol groups. In most cases, the EOF is greater than the electrophoretic flow which allows for it to be used as an internal pump. For many separations, however, the EOF must be suppressed or even eliminated. This can be done by increasing the buffer ionic strength, which decreases the zeta potential, or by modifying the silica surface.

1.3.5 Efficiency and Resolution in CE

The high voltages used in capillary electrophoresis result in increased efficiencies of the solute plug. Capillary efficiency, N, is reported as the number of theoretical plates (the same as N in liquid chromatography) and is related to the applied voltage, the solute's mobility, and its diffusion coefficient (D) by equation 1.15 [1]:

$$N = \frac{\mu V}{2D} \qquad (1.15)$$

CE has much higher efficiencies than high performance liquid chromatography. Plate counts have been reported as high as the 2-3 million range [18]. This is due mainly to the absence of a stationary phase which eliminates band broadening due to the resistance to mass transfer in the mobile and stationary phases. Another reason for the vast improvement in efficiency is that CE does not suffer from a parabolic, or laminar, flow profile. In HPLC, the pressure-driven mobile phase slows at the column walls because of frictional forces acting on the solvent molecules. This results in a parabolic flow gradient throughout the column. In CE, the profile of the electroosmotic flow is flat except very close to the capillary walls where the flow rate approaches zero. Figure 1.5 displays the differences in the two flow profiles:

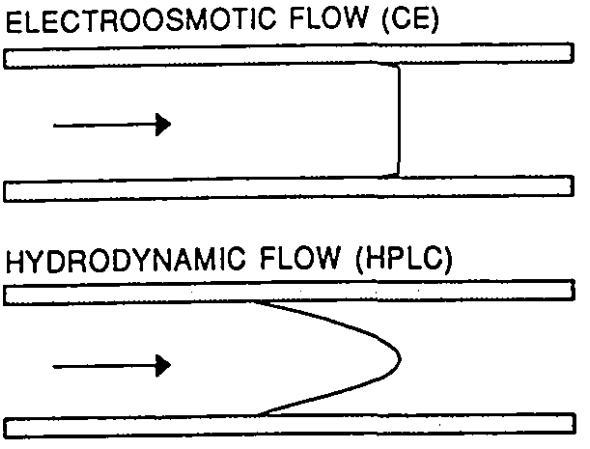

Figure 1.5 HPLC vs CE Flow Profiles

Another improvement in efficiency for CE is the absence of a pressure drop found in packed-column LC [14].

Capillary electrophoresis, however, does suffer from band broadening. The expression for efficiency in equation 1.15 is only a theoretical estimate of the number of plates. Band broadening in CE has several sources. Joule heating has the greatest effect on band broadening. It is due to the current passing through the capillary. The rate of heat production is a function of applied voltage:

$$dH/dt = \frac{kV^2}{L^2} \qquad (1.16)$$

Joule heating can be reduced by either decreasing the voltage or using a longer capillary. When the heat dissipates at the capillary wall, a temperature

gradient is formed and the differential can be a significant source of band broadening [19-21].

Other sources of band broadening include diffusion of the solute to an area of lower concentration, adsorption of the solute to the capillary walls (especially with basic compounds), injection plugs that are too long, and electromigration dispersion. The latter source is a result of differences in electrophoretic mobility between the solute and running buffer [22].

Resolution in CE, like in HPLC, is a measure of peak separation and is defined as:

$$R_s = \frac{1}{4} \frac{\Delta \mu_{ep} \sqrt{N}}{\mu_{avg} + \mu_{eo}} \qquad (1.17)$$

where $\Delta\mu$ is the difference in electrophoretic mobility of the two solutes and μ_{avg} is the average mobility of the two species. Substituting equation 1.15 into 1.17, where the value for μ is the overall mobility (i.e $\mu=\mu_{avg} + \mu_{eo}$), gives us a relationship between resolution, electrophoretic mobility, voltage, and diffusion coefficient:

$$R_s = 0.177 \Delta \mu_{ep} \sqrt{\frac{V}{(\mu_{avg} + \mu_{eo}) D_m}} \qquad (1.18)$$

The relationship between R_s and voltage indicates that increasing voltage is can be used as a way of increasing resolution; however large voltage increases also significantly increases band broadening due to Joule heating. Instead, the best way to increase resolution is to increase $\Delta\mu_{ep}$. This can be done by selecting the right mode of operation, the correct buffer composition and pH, and the use of buffer additives.

1.4 MODIFYING SELECTIVITIES IN CE AND HPLC

Both HPLC and CE use mobile phase and buffer additives, respectively, to provide specific solute interactions. A type of interaction that has become popular is the formation of inclusion complexes with cyclodextrin. These cyclic oligosaccharides complex with solutes with functional groups that fit into the cyclodextrin cavity. Cyclodextrins were first used in HPLC as a bonded stationary phase [23-26], however they have also been used as a mobile phase additive [27,28]. Cyclodextrins have also been used for inclusion complex formation in capillary electrophoresis as a buffer additive [29,30]. Cyclodextrins and their applications in HPLC and CE will be discussed in more detail in Chapter 2.

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CHAPTER 2

CYCLODEXTRINS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND CAPILLARY ELECTROPHORESIS

2.1 INTRODUCTION

Cyclodextrins, also known as Schardinger dextrins, cycloamyloses, and cycloglucans, are a class of toroidally shaped cyclic oligosaccharides produced from the enzymatic degradation of starch. Upon treatment with the amylase of *Bacillus macerans* (cyclodextrinase), the starch helix is partially hydrolysed and the resulting D(+)-glucopyranose unit chains are joined together by α-1,4 linkages [1,2]. Cyclodextrins were first discovered in 1891 by A. Villiers [3] but it was not until 1903 before the first detailed description of its preparation and isolation was reported by Schardinger [4].

The action of cyclodextrinase on starch is not very specific to the position of hydrolysis so the product of the reaction is a mixture of different sized cyclodextrins. α-, β, and γ- are the most predominant forms of cyclodextrin, each consisting of six, seven, and eight glucose units, respectively. These three forms, along with δ-cyclodextrin (9 glucose units), have all been isolated by a variety of selective precipitation reactions using suitable organic solvents [2,4-

11]. They have also been isolated by adsorption chromatography [12]. Cyclodextrins with greater than nine glucose units have been identified (ε-, ζ-, η-, and θ-cyclodextrin with 10, 11, 12, and 13 glucose units, respectively) but are produced in very small amounts [2,11]. Cyclodextrins having fewer than six glucose units are not known to exist due to steric hindrances [13] and the six-fold character of the starch helix [14].

Cyclodextrins have been the subject of an enormous amount of research in many areas of chemistry over the years and have been the subject of several books [15-21] and review articles [22-39]. This unique molecule interacts with other species by forming an inclusion complex with the cyclodextrin cavity. It was discovered that the formation of inclusion complexes could be used to catalyze reactions and that the cavity can be used as a model for enzyme active sites [40]. Modification of the cyclodextrin would change the cavity size and nature thus making it more selective for a specific guest. This ability to mimic enzyme-substrate interactions developed cyclodextrins into a valuable research tool.

Cyclodextrins have found many applications in analytical chemistry. These applications have been the subject of several reviews [21,34,39] and include the use of cyclodextrins as shift reagents and fluorescence enhancers with spectroscopic techniques, in electrochemical analysis as chemically modified electrodes, and in the separation sciences. The latter field initially was interested in cyclodextrins in the area of chiral separations. The chiral environment at the cyclodextrin rim groups allows them to distinguish between enantiomers of a suitable optically active molecule. Cyclodextrins have since become a powerful chiral selector in high performance liquid chromatography and capillary electrophoresis yet are also useful in the separation of non-chiral solutes.

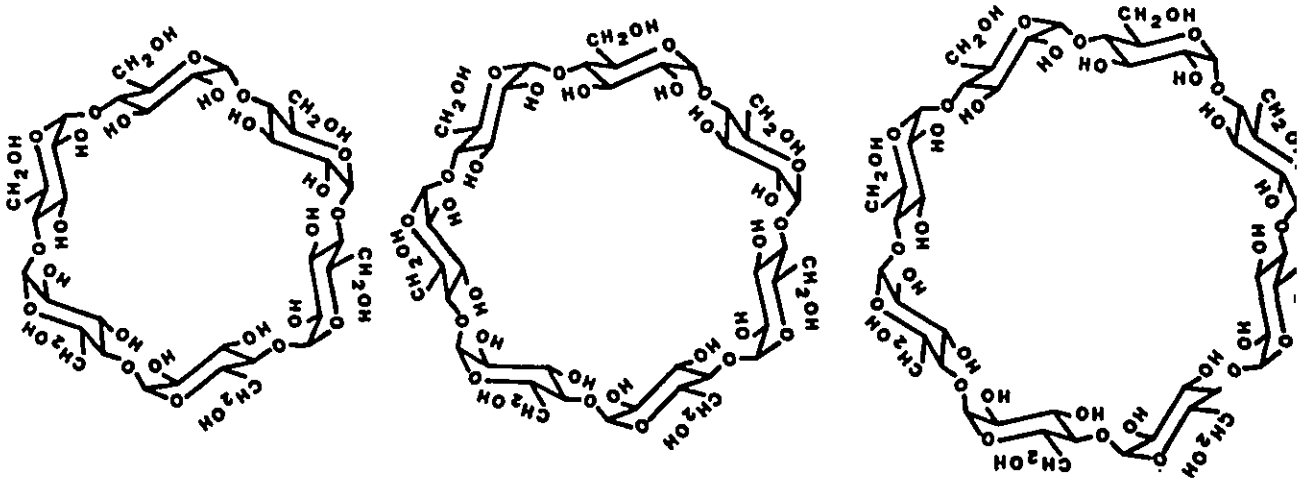
This chapter will focus on the uses of cyclodextrins in high performance

liquid chromatography and capillary electrophoresis. It will have a detailed description of the structure and physical properties of cyclodextrins followed by a literature review on cyclodextrins in HPLC and CE.

2.2 PROPERTIES OF CYCLODEXTRINS

2.2.1 Structure

Cyclodextrins are toroidally shaped with all the glucose units in a C1(D) chair conformation. The glucose units are joined by α-1,4 linkages and the size of the cyclodextrin depends on the number of glucose units it contains. Figure 2.1 shows a diagram for α-, β-, and γ-cyclodextrin and Figure 2.2 shows a side view of β-cyclodextrin.



α-Cyclodextrin β-Cyclodextrin γ-Cyclodextrin

Figure 2.1 Structure of α-, β-, and γ-Cyclodextrin

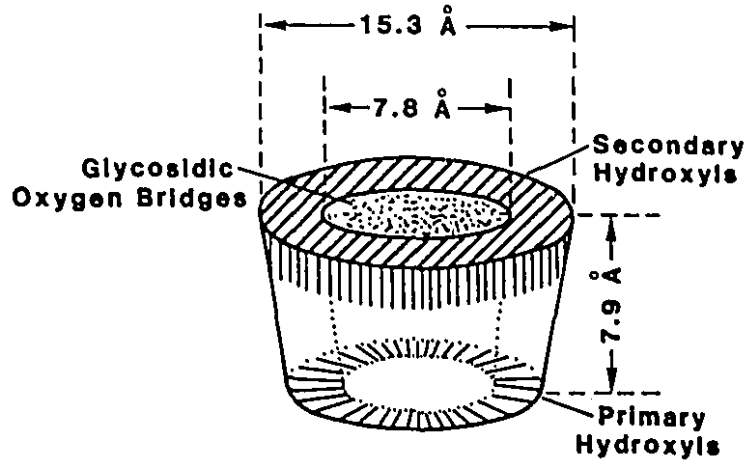


Figure 2.2 Side View Schematic of β-Cyclodextrin

The secondary hydroxyl groups (from the C-2 and C-3 atoms) are free to rotate and can be found at the larger end of the torus while the rigid primary hydroxyl groups from the C-6 atom are at the smaller end. These hydroxyl groups give the outside surface of the molecule a hydrophilic character. The cavity formed by the glucose units contains two rings of C-H groups with a ring of glucosidic oxygens in between. This results in a relatively hydrophobic cavity while the electron pairs from the glucosidic oxygens give the cavity some Lewis base character. Cyclodextrins have a rigid structure believed to be the result of an intramolecular hydrogen bonding network formed between the secondary hydroxyl groups at the larger end of the torus [41]. The hydrogen bonds are so strong that they exist in dimethylsulfoxide, a solvent that usually breaks the hydrogen bonds between two solutes [42].

2.2.2 Physical Properties

Cyclodextrins are stable compounds in solution at a pH greater than 3.5 which make them ideal for use under most HPLC and CE conditions. Below pH 3.5 cyclodextrins undergo partial hydrolysis to produce glucose and a series of non-cyclic maltosaccharides [15]. Also, there is no appreciable degradation of cyclodextrin by UV light.

Table 2.1 summarizes the physical properties of cyclodextrins.

TABLE 2.1
Physical Properties of Cyclodextrins[a]

Characteristic	Type of Cyclodextrin		
	α	β	γ
# Glucose Units	6	7	8
Molecular Weight	972	1135	1297
Water Solubility (g/100ml, 25°C)	14.5	1.85	23.2
Cavity Internal Diameter (Å)	4.7-5.3	6.0-6.5	7.5-8.3

[a] Data obtained from [39].

The two important physical properties of cyclodextrin to be considered in high performance liquid chromatography and capillary electrophoresis are their water solubilities and cavity internal diameters. In HPLC, cyclodextrins are used as both stationary phases and mobile phase additives. For the latter

methodology, water solubility is an important consideration when choosing the type of cyclodextrin needed for separation. The same consideration is necessary in CE as cyclodextrins are used as buffer additives. The cavity internal diameter is important since it determines the type of solutes that will be form inclusion complexes with the cyclodextrin.

2.2.3 Inclusion Complex Formation

The principal attribute of cyclodextrin is its ability to form inclusion complexes with various compounds where the guest molecules are incorporated into the cyclodextrin cavity. Inclusion complexation is reversible and has a relatively high rate of formation. Inclusion complexes usually have a 1:1 stoichiometry although 1:2 and 2:1 ratios do exist.

For an inclusion complex to form, the guest must fit into the cyclodextrin cavity. The complex can form with either the whole guest molecule or with suitable functional groups. The formation of the inclusion complex can be given by equation 2.1, where CD is the cyclodextrin, G is the guest, and CD-G is the inclusion complex:

$$CD + G \rightleftharpoons CD\text{-}G \qquad (2.1)$$

Inclusion complexes are dynamic species with the guest exchanging rapidly with free substrate molecules. The inclusion complex formation constant (K_f) is given by:

$$K_f = \frac{[CD\text{-}G]}{[CD]\ [G]} \qquad (2.2)$$

Further, the dissociation constant, K_d, can be expressed as:

$$K_d = \frac{1}{K_f} = \frac{[CD]\ [G]}{[CD\text{-}G]} \qquad (2.3)$$

On first observation, it appears that inclusion complex formation is based solely on hydrophobic interactions. Studies show that inclusion complexation is associated with favourable enthalpy change and either an unfavourable or slightly favourable entropy change [43-47]. However, hydrophobic interactions are usually associated with very favourable entropy changes [48-50] suggesting that inclusion complexation involves more than hydrophobic interactions. Several proposals have been made to explain some of the other binding forces behind inclusion complex formation:

a) Van der Waals interactions between the guest and host [43,51-53]. These include permanent dipole-induced interactions and London dispersive forces.

b) Hydrogen bonding between the guest and cyclodextrin hydroxyl groups [47,54-57].

c) The presence of high energy water molecules in the cavity while in aqueous solution [25,43]. This results from enthalpy-rich polar water molecules present in the cyclodextrin cavity that are unable to completely hydrogen bond with surrounding water molecules [58]. The favourable enthalpy change involved with replacing the water molecules is thought to be a driving force in inclusion complex formation.

d) Release of ring strain in the cyclodextrin. It has been found, by X-ray crystallography, that the strain energy of certain inclusion complexes is lower than the free cyclo-

dextrin [59-62]. However, this is not thought to be a major driving force in inclusion complexation [63].

The relative contributions of each force are difficult to calculate and are dependent on the nature of the guest molecule. Regardless of the forces, two characteristics are essential for inclusion complex formation: the guest must be of the right size and of the right geometry to fit into the cavity. A guest that is too large will not fit into the cavity (however parts of it may) and one that is too small will enter and exit the cavity without interacting with the cyclodextrin. A compound must be less polar than water to form an inclusion complex; however very hydrophillic compounds do not form strong inclusion complexes. Figure 2.3 is shows why orientation is important for inclusion complexation. Without proper orientation, inclusion complexation will not occur.

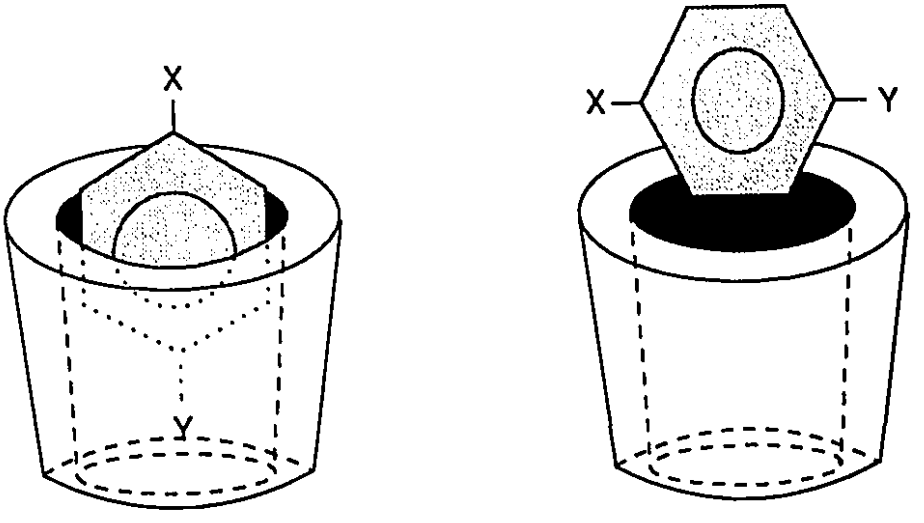


Figure 2.3 Schematic representation of inclusion complexation. (A) Solute with proper and (B) solute without proper orientation

Inclusion complexes usually require the presence of water; however they have been found to form in dimethylsulfoxide and dimethylformamide [64]. The presence of organic solvents in water will not suppress the formation of inclusion complexes; however the inclusion complex strength will weaken with decreasing water content.

The binding of cyclodextrin with substrates can be improved by modifying the cyclodextrin rim functional groups. Modification usually involves either replacing just the H atom from the hydroxyl groups or replacing the entire hydroxyl group. This leads to a less polar cavity and stronger inclusion complexes.

One characteristic of inclusion complexes is their ability to distinguish between enantiomers of suitable guests. Optical isomers have identical physical properties in an achiral environment; however a suitable chiral reagent will be able to distinguish between the two. Each glucose unit of the cyclodextrin contributes five chiral centres resulting in a highly enantioselective site for chiral recognition at the rim hydroxyl groups. The mechanism for chiral recognition requires that the guest enter the cavity in a manner that will allow the asymmetric center to interact with the hydroxyl groups at the cavity rim.

If there is no association with the chiral rim groups, there will be no enantioselectivity. The ability for cyclodextrin to distinguish between enantiomers does not necessarily depend on the extent of penetration into the cavity but rather on the degree of interaction between the substrate and chiral hydroxyl groups [65]. Enantioselectivity is based on differences in inclusion complex strength for each isomer; therefore the binding forces for each isomer must be different or else the cyclodextrin will not be able to distinguish between them.

Cyclodextrins' ability to form inclusion complexes has several uses in industry. They are used as solubilizing and complexing agents in the food,

cosmetic, and pharmaceutical industries. As well, they are used in the chemical industry as catalysts for improving selectivity and in the separation and purification of end-products [39]. Also, as was mentioned, they are used for chromatographic separations and the remainder of this chapter will be a literature review on the use of cyclodextrins in high performance liquid chromatography and capillary electrophoresis.

2.3 CYCLODEXTRINS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Cyclodextrins have found many uses in the area of chromatographic separations. Their remarkable ability to differentiate between solutes, especially optical, structural, and geometric isomers have made them an important tool in most types of chromatography. Due to its size, β-cyclodextrin is by far the most popular of the cyclodextrins, although the smaller α-cyclodextrin and the larger γ-cyclodextrin do find some application where the size of the solute is a factor.

Cyclodextrins are useful for more than just HPLC. To briefly discuss their other uses, cyclodextrins have been used as a mobile phase additive in thin-layer chromatography in the separation of a large host of solutes. To name a few, α-cyclodextrin has been used in the separation of phenols [66,67] and amino acid enantiomers [68,69,70] while a variety of chiral drugs, such as mephenytoin and benzylnornicotine, were separated using β-cyclodextrin [70]. More water-soluble β-cyclodextrin derivatives have been used in the separation of amino acid derivatives using matosyl-β-cyclodextrin [71] as well as both hydroxyethyl- and hydroxymethyl-β-cyclodextrin [72]. Cyclodextrins have also been used as a stationary phase in high performance thin-layer chromatogra-

phy for the separation of a variety of isomers [73].

Cyclodextrins have also been immobilized onto a support and used in affinity chromatography for the analysis of proteins. Uppsala [74] separated α- and β-amylase with α-cyclodextrin while others have reported the separation of starch debranching enzymes [75] and fibroblast growth factor (FGF) [76].

Cyclodextrins, both derivatized and underivatized, have also found great use as a stationary phase in gas chromatography. They have been used in the separation of a variety of structurally related compounds [77-82] and enantiomers [83-87].

However, it is in high performance liquid chromatography that cyclodextrins have found the greatest application. Here, they are used as a stationary phase or as a mobile phase additive.

2.3.1 Cyclodextrin Stationary Phases

Cyclodextrin stationary phases were first produced in the mid-1970's by the formation of polymerized and crosslinked gels, based on immobilized resins first described by Solms and Egli in 1965 [88]. They cross linked a mixture of α-, β-, and γ-cyclodextrin with epichlorhydrin to form glyceryl bridges between the cyclodextrin units. Researchers in the mid-1970's immobilized the cyclodextrin-polymer gel to produce a liquid chromatography column. Other resins were also tested, such as cyclodextrin-polyurethane and cyclodextrin-poly(vinyl alcohol). These gels were used in a variety of separations, such a amino acids [89], nucleic acids [90], alkaloid enantiomers [91,92], and warfarin enantiomers [93]. Polymer-cyclodextrin gel stationary phases, however, are not suitable for HPLC. The soft gels have poor mechanical strength in the presence of the high pressures found in HPLC. As well, the gels suffer from poor separation efficiency as the solute must travel to the interior of the polymer particle rather than just interact with the particle surface. The increased time needed

for this diffusion-controlled process leads to poor column efficiency.

These shortcomings of cyclodextrin gels led researchers to seek ways of developing more stable stationary phases. Research focused on anchoring cyclodextrins to silica gel through propylamine, ethylene diamine, and other similarly-structured linkages [94-98]. These new phases were still rather unstable, though, and cyclodextrin loading efficiency to the silica was low. Another problem was that the amines present in the spacer arm tended to react with the solutes. In 1984, Armstrong developed the first cyclodextrin stationary phase containing no nitrogen-based linkages [99]. The cyclodextrins were bonded to silica gel with a 6-10 atom spacer. This innovative method resulted in a stable stationary phase that could be used in the reversed-phase mode. Armstrong and co-workers [100] first reported using this new phase in the separation of polycyclic aromatic hydrocarbons, quinones, mycotoxins, and heterocyclic compounds. As well, a wide variety of structural isomers were separated with the improved cyclodextrin phases, including phenols, derivatized benzoic acids, cis/trans derivatized benzene, and various steroid epimers [101]. Figure 2.4 shows the separation of benzo[e]pyrene and benzo(a)pyrene on a β-cyclodextrin column.

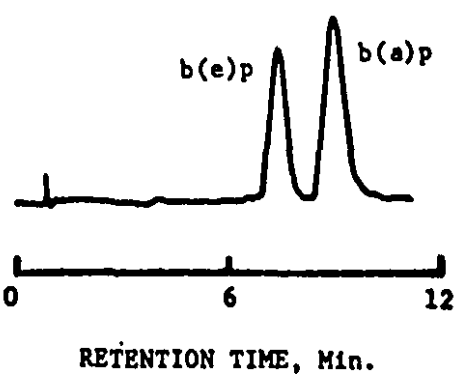


Figure 2.4 Separation of benzo[e]pyrene and benzo[a]pyrene. Mobile phase: 50:50 methanol:water [101].

These new cyclodextrin columns also proved to be suitable for the separation of optical isomers. Hinze and co-workers [102] used a β-cyclodextrin column to separate a number of enantiomers, such as derivatized amino acids, barbiturates, substituted phenyl acetic acid, and dioxalanes. Figure 2.5 shows the separation of hexobarbital enantiomers as a function of organic modifier.

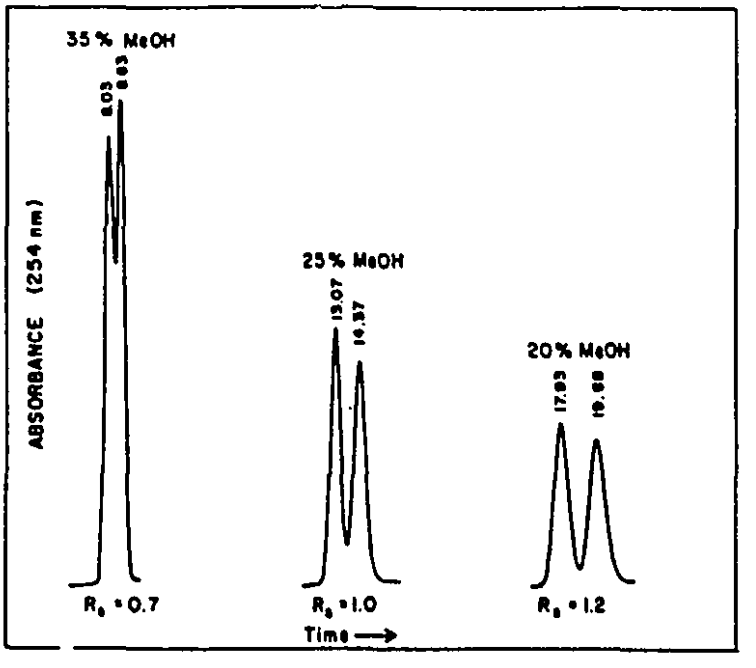


Figure 2.5 Resolution of hexobarbital enantiomers on a β-cyclodextrin column as a function of methanol concentration in the mobile phase [102].

Enantioselectivity increased as the methanol concentration decreased due to stronger inclusion complexes. Other chiral separations include, to name but a few, the resolution of optical isomers of metallocene [103], crown ethers [104,105], mandelic acid derivatives [106], scopolamine, cocaine, humatropine, and atropine [107], ibuprofen metabolites [108], dinitrophenyl amino acids [109], β-blockers [110], nipecotic acid amides [111], aromatic cyclic peptides [112], and ephedrine [113]. Much research is still being carried out using cyclodextrins in the separation of optical isomers.

Considerable research has been carried out using cyclodextrin stationary phases in the separation of other types of isomers and structurally-related compounds. Research in this area includes the separation of cis/trans prostaglandins [114], dipeptides [115-118], biphenyl metabolites [119], substituted anilines [120,121], pyridine derivatives [122], saccharides [123-126], phenothiazines [127], chlorophenols [128-130], and the fullerenes C_{60} and C_{70} using a γ-cyclodextrin column [131].

A significant amount of work has also been done in improving selectivity via derivatization of the cyclodextrin rim functional groups. This broadens the applicability of cyclodextrin stationary phases as it introduces new interactions between the rim groups and the solutes. These new phases are used for the same types of separations as underivatized cyclodextrin; however derivatization has been found, in some cases, to improve separation or provide separation that was not possible with the underivatized cyclodextrin [132-135].

2.3.2 Cyclodextrin Mobile Phases

An alternative to cyclodextrin stationary phases is to use them as mobile phase additives while using a conventional stationary phase, such as octadecylsilane (C_{18}). Cyclodextrins have several characteristics that make them suitable as mobile phase additives:

a) They form reversible and selective inclusion complexes.

b) The inclusion complexes have relatively high rates of formation.

c) Cyclodextrins are stable over a large pH range.

d) They do not absorb UV light.

e) They are non-toxic.

Cyclodextrin mobile phases, however, have several drawbacks:

a) The main disadvantage of cyclodextrins, especially β-cyclodextrin, are their relatively low solubility in aqueous systems.

b) They may contribute to band broadening since there can be resistance to mass transfer between the additive and stationary phase and between the additive and solute [136].

c) If the separation is on a preparative scale, the cyclodextrin must be removed and the solute purified.

The resolution of solutes using cyclodextrin mobile phases depends on several factors:

a) Differences in the stability constants of the cyclodextrin/solute complexes.

b) Differences in the adsorption of the inclusion complexes on the surface of hydrophobic stationary phases.

c) Differences in the adsorption of free solute molecules on the cyclodextrin layer adsorbed on the reverse-phase surface.

Cyclodextrin additives are usually used in the reverse-phase mode as they require the presence of water for inclusion complexation. Because of their selectivity and much lower price, β-cyclodextrin is a more popular additive than α- or γ-cyclodextrin. All types of underivatized cyclodextrins (α-,β-,and γ-) are weakly adsorbed onto C_{18} columns, but their more non-polar derivatives may be adsorbed quite strongly.

Complexation and adsorption equilibria for cyclodextrin-containing mobile phases are very complicated. This is because many species of one solute can be complexed (neutral, ionized, free) which makes it difficult to predict retention. The three-phase equilibrium model for the partitioning of the solute between the bulk mobile phase and dissolved cyclodextrin is illustrated in Figure 2.6 [137].

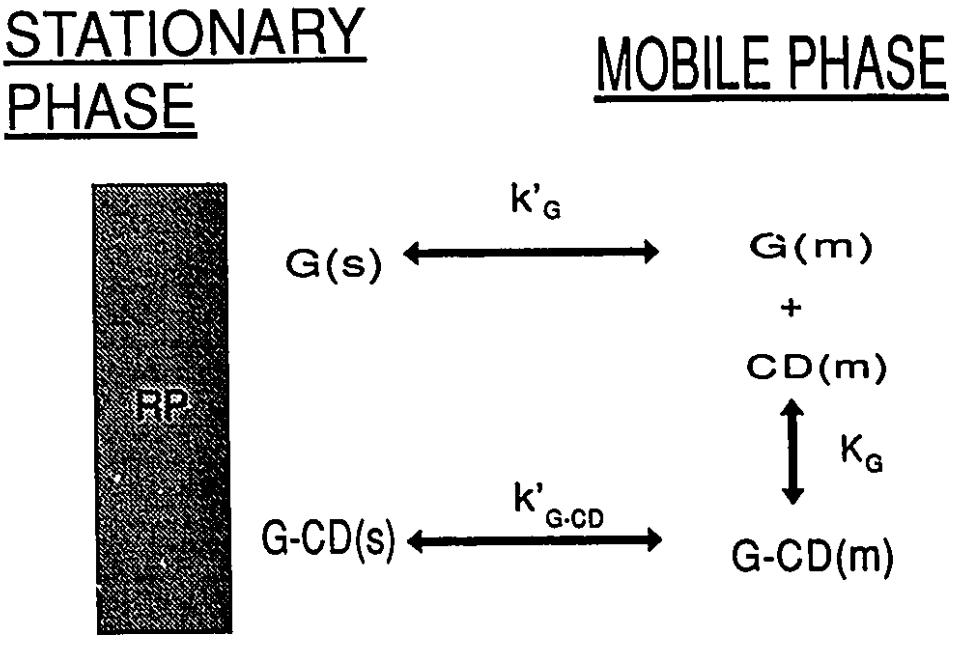


Figure 2.6 Three phase equilibrium model for cyclodextrins in the mobile phase.

G represents the guest solute, CD the cyclodextrin molecule, k'_G and $k'_{G\text{-}CD}$ are the capacity factors for the free guest molecule and inclusion complex, respectively, and K_G the stability constant of the G-CD complex having a 1:1 stoichiometry. The stability constant, which is the equilibrium constant for the inclusion complex in the mobile phase, can be given by:

$$K_G = \frac{[G\text{-}CD]_m}{[G]_m [CD]_m} \qquad (2.4)$$

The stability constant can also be defined as the ratio of the entrance and exit rate constants between the solute and the cyclodextrin. A small amount of organic modifier added to an aqueous mobile phase increases the value of K_G due to the displacement of water molecules from the cavity. But above about 10% organic modifier, the decrease in solvent polarity results in an increase in the solubility of the solute thereby decreasing the likelihood of the formation of an inclusion complex (i.e. a decrease in K_G). The greater the value of K_G, the greater the strength of interaction and therefore the greater the rate of decrease in the apparent capacity factor, k'_{app} with increasing cyclodextrin concentration [138].

The capacity factor for the free guest molecule, can be given by:

$$k'_G = \frac{[G]_s}{[G]_m} \qquad (2.5)$$

where $[G]_s$ and $[G]_m$ are the concentration of guest molecule in the stationary and mobile phase, respectively. The capacity factor for the inclusion complex, k', can be similarly described as:

$$k'_{G\text{-}CD} = \frac{[G\text{-}CD]_s}{[G\text{-}CD]_m} \qquad (2.6)$$

The apparent capacity factor for a given solute will be the sum of the

concentrations of the guest molecule and of the inclusion complex in both the stationary and mobile phase:

$$k'_{app} = \frac{[G]_s + [G\text{-}CD]_s}{[G]_m + [G\text{-}CD]_m} \qquad (2.7)$$

Substituting equations 2.4, 2.5, and 2.6 into equation 2.7 gives us the relationship between the apparent capacity factor and the concentration of cyclodextrin in the mobile phase:

$$k'_{app} = \frac{k'_G + k'_{G\text{-}CD} * K_G[CD]}{1 + K_G[CD]} \qquad (2.8)$$

Cyclodextrins have been used in the mobile phase to perform a variety of chiral separations, almost always in the reverse phase. Figure 2.7 shows an early separation by Debowski and co-workers, who used β-cyclodextrin to separate mandelic acid enantiomers [139].

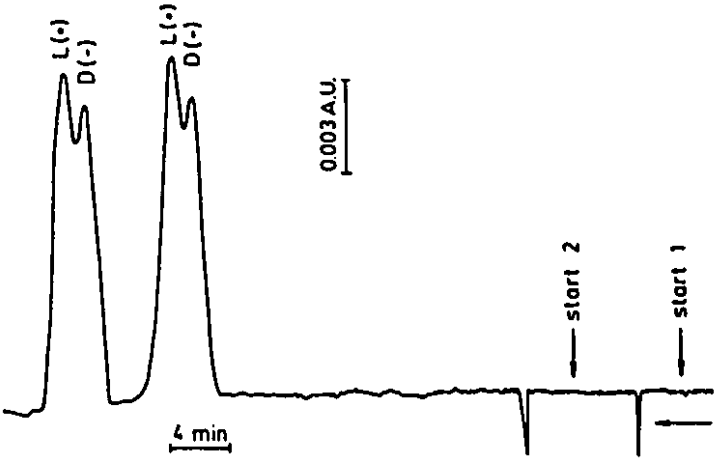


Figure 2.7 Separation of mandelic acid enantiomers. Mobile phase: 0.1M Phosphate, pH 2.1, 5mM β-cyclodextrin [139].

This chromatogram shows two consecutive injections of the same mixture. Figure 2.8 is the separation of 1-[2-(3-hydroxyphenyl)-1-phenylethyl]-4-(3-methyl-2-butenyl)piperazine using β-cyclodextrin in the mobile phase on a C_{18} column [140].

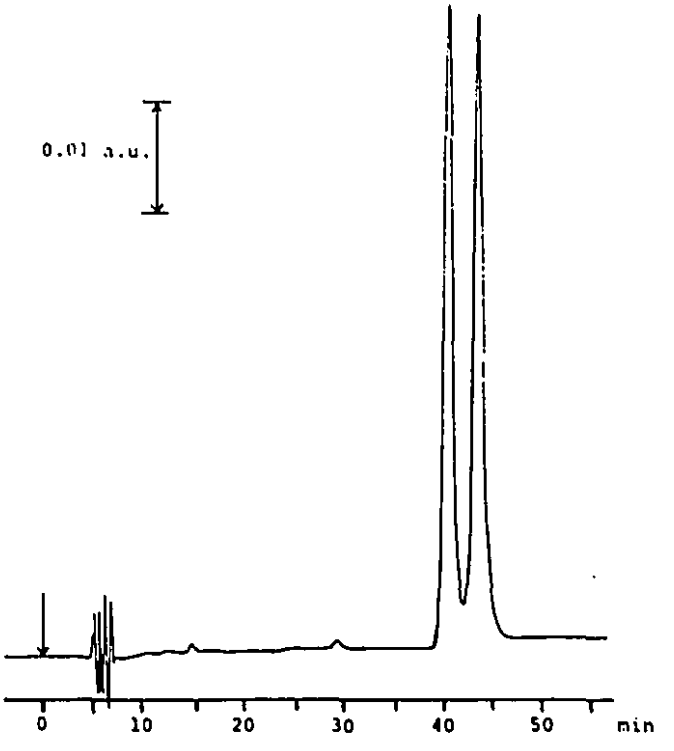


Figure 2.8 Enantiomeric resolution of 1-[2-(3-hydroxyphenyl)-1-phenylethyl]-4-(3-methyl-2-butenyl) piperazine. Mobile phase: 82:12:4 0.1M acetate:ethanol:β-cyclodextrin [140].

Other chiral compounds separated with β-cyclodextrin mobile phases include sorberol [141], norgestrel [142,143], derivatized amino acids [144,145], trimeprazine [146], and flumecinol [147]. In the separation of ephedrine, Mularz and co-workers [148], using a β-cyclodextrin mobile phase with a cyanopropyl column, could separate ephedrine enantiomers but not those of pseudoephedrine. Ephedrine and pseudoephedrine are diastereomers with the variation occuring in the position of a hydroxyl group. ^{1}H NMR was used to confirm that an inclusion complex was formed with ephedrine but not with pseudoephedrine as inclusion complexation will cause shifts in the NMR spectrum. This shows the sensitivity that β-cyclodextrin has towards solute structure. Even a slight difference in structure can effect the formation of an inclusion complex and thus enantioselectivity.

Modified β-cyclodextrins have also been used as mobile phase additives. As was previously mentioned, modification comprises of replacing just the H atom from the hydroxyl groups or by replacing the entire hydroxyl group with another functional group. This increases the hydrophobicity of the cavity and allows for tighter inclusion complexes as well as provides for different solute-cyclodextrin interactions. Another advantage is that modification tends to increase the solubility of the cyclodextrin making it more useful as a mobile phase additive. The most common modification is to replace the hydroxyl groups with methyl groups; however hydroxyethyl and carboxymethyl substitutions have also been reported. Zukowski and co-workers [149,150] used modified β-cyclodextrins with methyl groups in the resolution of barbiturate enantiomers while Pawlowska and Lipkowski [151] describe the separation of decyl benzene, 2,2'-dihydroxy-1,1'-binapthol, morsuximide, methylphenobarbital, glutethimide, methylmandelate, and mephenytoin enantiomers. The authors dynamically coated a silica stationary phase with trimethyl-β-cyclodextrin (TM-β-CD) by adding it to the mobile phase and then removing it.

They compared the enantioselectivity with and without additional TM-β-CD in the mobile phase. Figure 2.9 shows the separation of methylphenobarbital enantiomers. Enantioselectivity was observed with only the dynamically coated stationary phase; however there was superior resolution with the addition of cyclodextrin to the mobile phase.

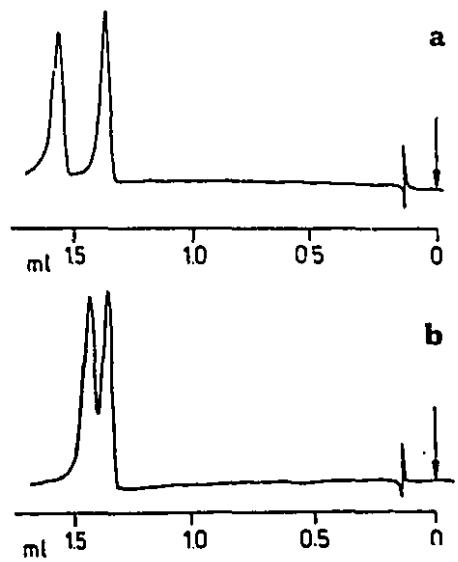


Figure 2.9 Enantiomeric separation of methylphenobarbital with (a) 65 mg/ml TM-β-cyclodextrin in the mobile phase and a dynamically coated column and (b) dynamically coated column and no TM-β-cyclodextrin in the mobile phase [151].

Cyclodextrins in the mobile phase, as was the case with cyclodextrin stationary phases, are also useful for non-chiral separations. Sybilska and co-workers [152] used α- and β-cyclodextrin in the mobile phase for the separation of o-,m-, and p-nitro-cis- and trans-cinnamic acids. Figure 2.10 shows separation of all six isomers. It is clear that cyclodextrin can differentiate between structural isomers; however the authors attribute the incomplete separation to poor column efficiency.

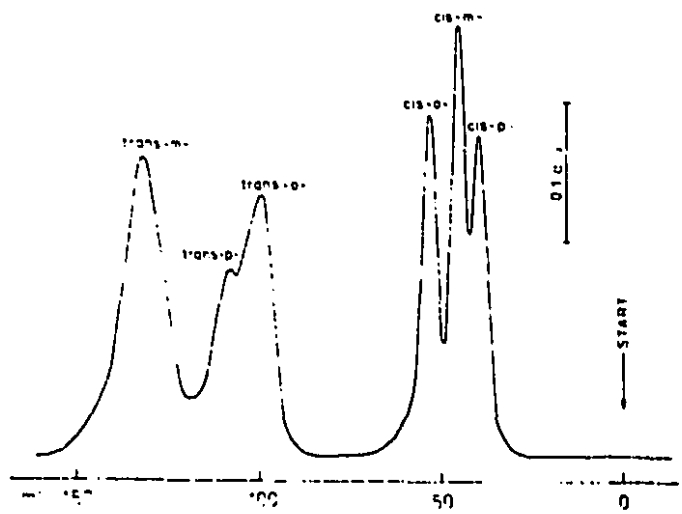


Figure 2.10 Separation of *cis-trans* mixture of o-, m-, and p-nitrocinnamic acid. Mobile phase: 0.095M sulfate buffer, pH 2, 4mM β-cyclodextrin [152].

Other examples of non-chiral separations include pyridine derivatives [153], estriol metabolites [154], NADH and NADP [155], polycyclic aromatic hydrocarbons with β-cyclodextrin [156] and γ-cyclodextrin [157], aflatoxins [158], and bile acids and salts [159,160].

Some work has been carried out on characterizing the inclusion complex formed with β-cyclodextrin mobile phases. Fujimura and co-workers [161] studied the effect of the organic modifier and cyclodextrin concentration on inclusion complex strength and thus the capacity factor of aromatic compounds. They found that increasing the water content in the mobile phase increased the capacity factor while increasing cyclodextrin concentration decreased it. Mohseni and Hurtubise [162] studied the changes in standard enthalpy and entropy of hydroxyl aromatics when β-cyclodextrin was added to the mobile phase. They concluded that the addition of cyclodextrin increased the system entropy (i.e. ΔS) and decreased enthalpy (ΔH) which implies the solutes favoured interaction with the stationary phase relative to the β-cyclodextrin cavity.

Finally, a few reports have been published describing the use of cyclodextrin mobile phases for semi-preparative separations. Cyclodextrin mobile phases are not well suited for preparative chromatography as the cyclodextrin must be isolated from the solute. Harada an co-workers [163] used α-cyclodextrin to separate ferrocene deravitive enantiomers while Cooper and Jeffries [164] used β-cyclodextrin to separate brompheniramine enantiomers. In the latter work, the authors achieved a throughput of 8 mg per hour for each enantiomer. They then used a polystyrene-divinylbenzene column to isolate the solute from the cyclodextrin and buffer molecules present in the mobile phase.

Cyclodextrins can also be used to enhance fluorescence detection of certain solutes. Inclusion complexation can cause significant shifts in emission

wavelengths making cyclodextrin beneficial for both separation and detection [165]. A six-fold increase in fluorescence signal was reported for 5-methoxy-sporalen with β-cyclodextrin in the mobile phase [166] and Shimada and co-workers [167] recently reported the analysis of five 20-oxosteroids using fluorescence detection with cyclodextrin as a mobile phase additive.

2.4 CYCLODEXTRINS IN CAPILLARY ELECTROPHORESIS

Cyclodextrins have been used successfully in the capillary electrophoretic separation of enantiomers, structural isomers, and other structurally-related compounds. Widespread use of cyclodextrins in CE began in 1991 and research in this field has grown exponentially since then. Until that time, cyclodextrins were used predominantly as a chiral selector for enantiomeric separations. A great deal of research is still conducted on chiral separations, however cyclodextrins are presently finding a lot of applications for non-chiral separations.

2.4.1 Cyclodextrins in Capillary Zone Electrophoresis

In the capillary zone mode of electrophoresis (CZE), where the background electrolyte is homogenous and contains no gel or pseudo-phase, the solute must be charged as neutral cyclodextrin will migrate only with the electroosmotic flow. Separation is based on differences in partitioning between the cyclodextrin cavity and the run buffer. Resolution occurs when a suitable solute enters the cyclodextrin cavity, migrates a short time as an inclusion complex, and then exits to continue its electrophoretic migration. Neutral cyclodextrin will retard anionic solutes and assist in the migration of cationic solutes.

Enantiomeric separations have been achieved in capillary zone electrophoresis with cyclodextrins as a buffer additive. Fanali [168,169] used

heptakis(2,6-di-O-methyl)-β-cyclodextrin for the separation of ephedrine, norephedrin, epinephrine, norepinephrine, isoproterenol, terbutaline, and propranolol enantiomers. Tanaka and co-workers [170] separated dansylamino acid enantiomers with β-cyclodextrins but found that methylation of the cyclodextrin reduced enantioselectivity. Otsuka and Terabe [171] separated the enantiomers of chlorpheniramine on a 26 cm, 50 μm I.D. capillary. The electropherogram of this separation can be seen in Figure 2.11. This diagram demonstrates the exceptional efficiency one gains with capillary zone electrophoresis. Other chiral separations performed with cyclodextrin, to name a few, include binapthyl amino acids [172], β-amino alcohols [173], warfarin in plasma [174], mandelic acid [175], and salbutamol and its impurities [176].

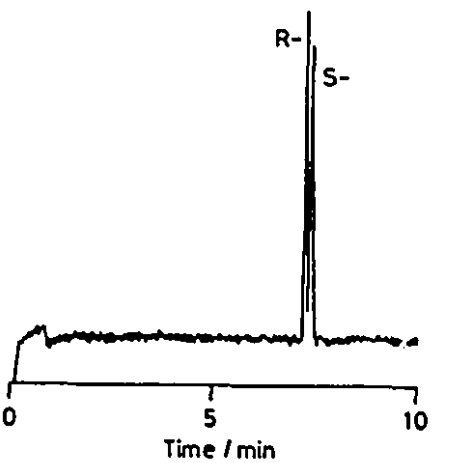


Figure 2.11 Optical resolution of RS-chlorpheniramine. Buffer: 5M urea, pH 3.0, V=10kV, 100mM β-cyclodextrin [171].

Cyclodextrins have also been used in CZE for non-chiral separations, including the separation of nucleotide isomers with the aid of borate complexation [177], gangliosides [178], 13-cis/trans retinoic acid [179], and plant growth regulators [180].

2.4.2 Cyclodextrins in Micellar Electrokinetic Chromatography

Another common methodology in CE is to use cyclodextrin in combination with charged micelles in a subset of capillary electrophoresis called micellar electrokinetic chromatography (MEKC). MEKC differs from capillary zone electrophoresis in that it employs a charged micellar system which acts as a pseudo-stationary phase. Micelles, also called surfactants, are molecules which consist of a long hydrophobic tail and a hydrophillic head. Above a certain concentration in solution, called the critical micelle concentration (CMC), these molecules spontaneously rearrange themselves such that the hydrophobic tails come together and the ionic heads point outward to form a roughly spherical aggregate. A schematic of a micelle can be seen in Figure 2.12. The impetus for aggregation is the lowering of the overall free energy of the system caused by the accumulation of hydrophillic and hydrophobic moieties. The beneficial quality of micelles is their ability to dissolve water-insoluble molecules by interaction with the hydrophobic center of the micelle. This characteristic has made micelles useful in both high performance liquid chromatography and capillary electrophoresis. In MEKC, uncharged solutes now can be separated providing they interact distinctly with the micelles. The most common surfactants used in CE are the anionic sodium dodecyl sulfate (SDS) and sodium cholate (SC). In untreated capillaries, these micelles travel towards the anode, opposite of the electroosmotic flow thereby retarding the migration of uncharged solutes travelling with the EOF. Cationic surfactants, such as cetyltrimethyl ammonium bromide (CTAB), also find use in CE.

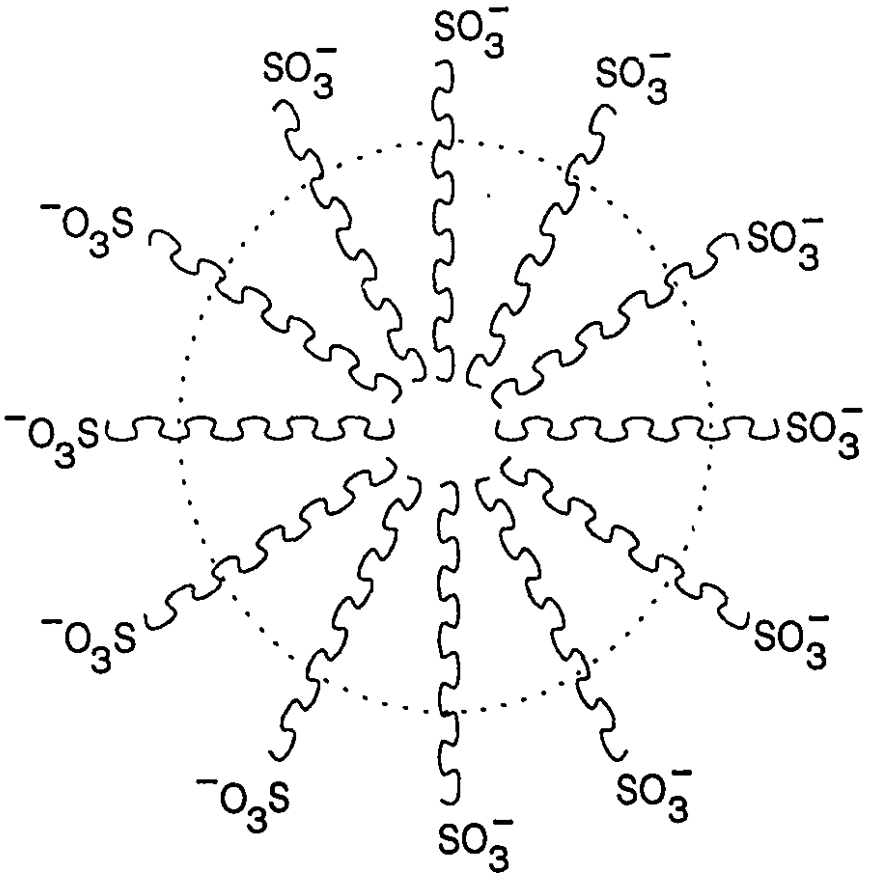


Figure 2.12 Micellar structure

They travel in the same direction as the EOF and assist in solute transport.

When cyclodextrins are added to the micellar system, they migrate with the electroosmotic flow with the solute partitioning between the micelle and cyclodextrin, as shown in Figure 2.13.

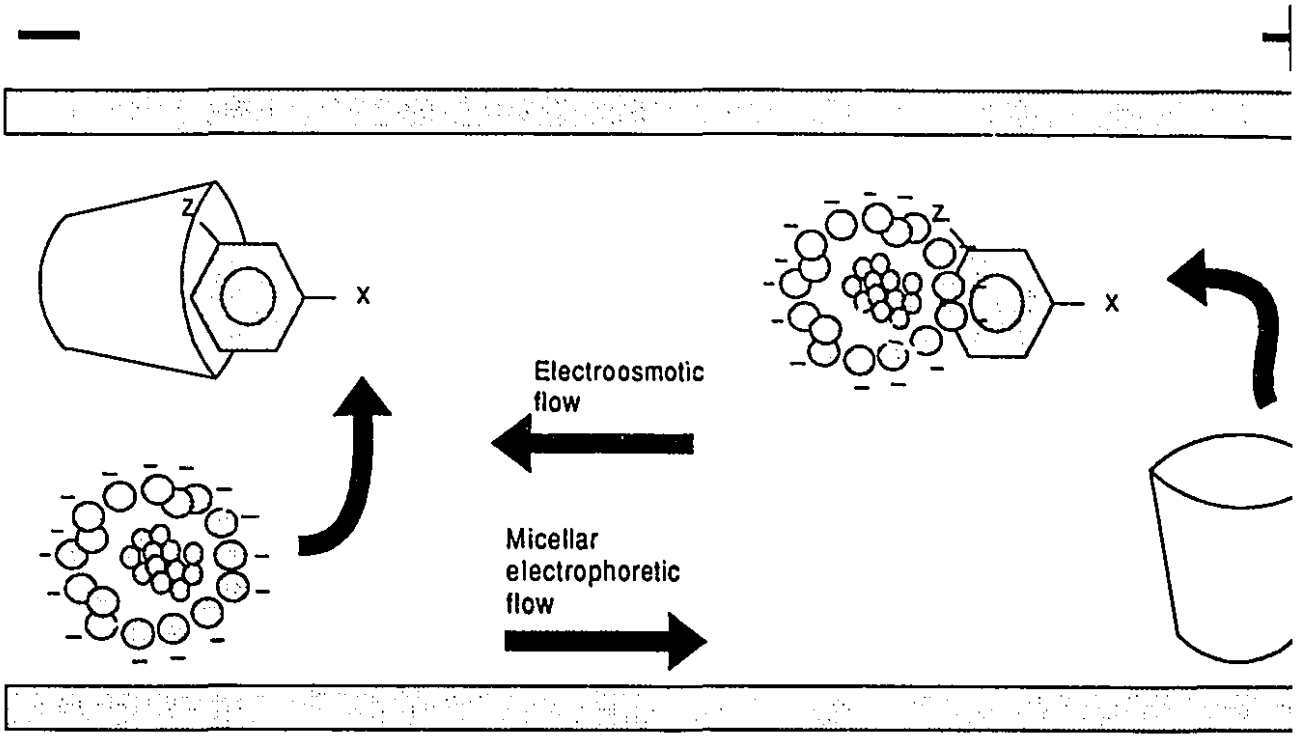


Figure 2.13 Mechanism in Cyclodextrin-MEKC

Ideally, micelles and cyclodextrin should have little interaction with each other, therefore it is unnecessary to account for CD-guest interactions with the pseudo-phase as one has to do with cyclodextrin mobile phase additives in HPLC. Increasing the cyclodextrin concentration will decrease a solute's migration time while increasing the anionic micellar concentration will increase the time so a balance must be established to optimize resolution.

The majority of research involving cyclodextrins in CE has been in the area of MEKC. The two reagents work well together to separate solutes that would otherwise be unresolvable by CE. Enantiomeric separation by MEKC with cyclodextrins has not found wide applicability and in fact most chiral separations are preformed by CZE. This is probably due to the decrease in efficiency with the micelles as compared to CZE. In MEKC, band broadening is more significant than in CZE due to the resistance to mass transfer into and out of the micelle and thermal gradients within the micelle system [181]. Examples of chiral separations by MEKC with cyclodextrin include dansyl-DL-amino acids [182], cicletanine [183], some chiral barbiturates [184], and for both diniconazole and uniconazole [185].

Cyclodextrin-MEKC has proven to be much more useful in the separation of structurally related compounds. In many cases, micelles are not selective enough to distinguish between molecules with similar structures so cyclodextrins are employed as another selector to aid with resolution. Copper and Sepaniak [186] were not able to resolve a series of benzopyrene isomers using just SDS or sodium cholate. Upon the addition of γ-cyclodextrin, they were able to separate a series of seven benzopyrenes (BaP). An electropherogram can be seen in figure 2.14.

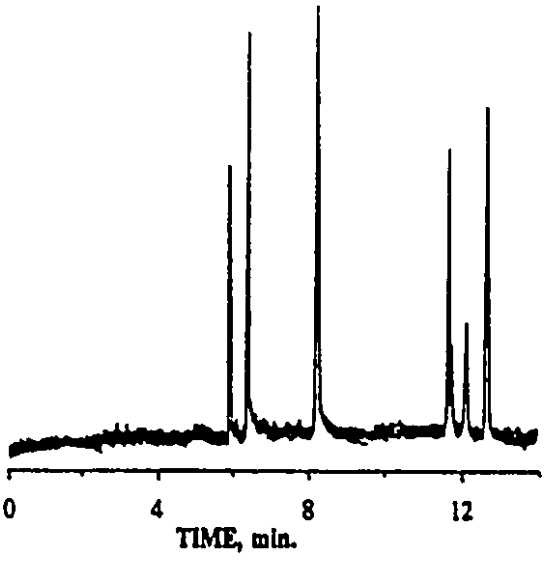


Figure 2.14 Separation of methyl substituted benzopyrene isomers. The elution order is BaP, 3-Me BaP, 5-Me BaP, 7-Me BaP, 1-Me BaP, 10-Me BaP, and 6-Me BaP. Buffer: 20 mM SDS, 15 mM γ-cyclodextrin, 5% (v/v) 2-propanol [186].

The authors also found that derivatizing the γ-cyclodextrin decreased selectivity and postulated that this was due to a less rigid structure for the modified cyclodextrin. Other examples of non-chiral separations with cyclodextrins in MEKC include polycyclic aromatic hydrocarbons [187,188], tetracyclic antibiotics [189], estrogens [190], a mixture of seven water-soluble and two fat-soluble vitamins [191], and opium alkaloids [192].

2.4.3 Charged Cyclodextrins in Capillary Electrophoresis

A third strategy employs ionic cyclodextrin in either CZE or MEKC. The rim groups are replaced with chargeable functional groups, such as a carboxymethyl or sulfobutyl ether group, so that cyclodextrin and its inclusion complex will migrate electrophoretically. The hydrophobic dependency of the inclusion complex makes charged cyclodextrins act almost as ionic micelles but with the added ability to distinguish between enantiomers and small differences in structure. Charged cyclodextrins are suitable for the analysis of both charged and neutral solutes.

To date, little reseach has been carried out using ionic cyclodextrins, and most of that work has focussed on chiral separations. Chankvetadze and co-workers [193] separated the enantiomers of some binapthyl derivatives using carboxymethyl-, sulfoethyl ether-, and sulfobutyl ether-β-cyclodextrin. The same research group used sulfobutyl ester β-cyclodextrin in the separation of thalidomide enantiomers [194] using MEKC. Gahm and Stalcup [195] used 1-(1-naphthyl)ethylcarbamoylated β-cyclodextrin in CZE for the separation of dinitrobenzoyl amino acid enanantiomers. Sulfobutyl ester-β-cyclodextrin was used in the separation of enantiomers of ephedrine and pseudoephedrine [196] and a variety of basic drugs, such as amphetamine, methamphetamine, and cocaine [197].

There has been limited research dealing with ionic cyclodextrins in non-chiral separations. Terabe and co-workers [198] were the first to employ ionic cyclodextrins in CZE where they used carboxymethyl-β-cyclodextrin (CM-β-CD) in the separation of some substituted benzenes. Figure 2.15 shows the separation of 6 xylidine isomers.

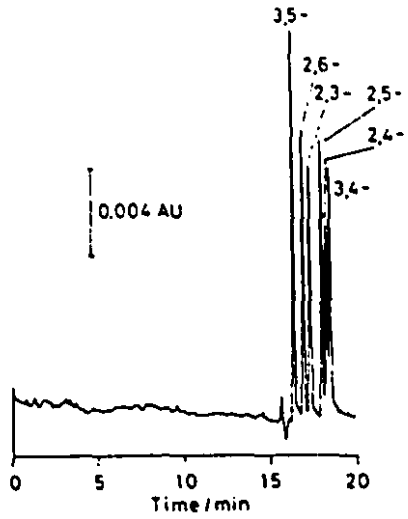


Figure 2.15 Separation of xylidine isomers. Buffer: 0.1M phosphate buffer, pH 7.0, 25 mM CM-β-CD [198].

Note the difference in efficiency between capillary electrophoresis and high performance liquid chromatography. Compare, for example, Figures 2.10 and 2.15. Even after a run time of more than 15 minutes, the peak shape is sharp and symmetrical, unlike HPLC whose band broadening mechanisms will never allow the efficiencies in high performance liquid chromatography to compare to those of capillary electrophoresis. In another example, Smith [199] separated ortho-, para-, and meta-nitrobenzyl alcohol in CZE with 25mM carboxymethyl-β-cyclodextrin in the buffer at a pH of 8.4.

The potential of charged cyclodextrin in capillary electrophoresis has not been exploited yet. The main drawback to ionic cyclodextrins is their high cost

relative to the underivatized molecule. However, since CE buffers requires a small amount of additive compared to HPLC, there is much potential for the future use of ionic cyclodextins in capillary electrophoresis.

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CHAPTER 3

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF DERIVATIZED AMINO ACID ENANTIOMERS USING MODIFIED β-CYCLODEXTRIN AS A MOBILE PHASE ADDITIVE

3.1 INTRODUCTION

A molecule has the property of chirality when the mirror image of its structure is non-superimposable on itself. A pair of non-superimposable isomers are referred to as enantiomers, or optical isomers. The most common structural feature of a chiral compound is the presence of an asymmetric centre consisting of an atom with four different atoms or functional groups bonded to it. The most prevalent asymmetric centre is an sp^3 carbon atom accommodating four different substituents, although other asymmetric centres are possible, such as nitrogen in quaternary ammonium compounds, cyclic sulphoxides, and pentavalent phosphorous compounds. Chirality can also exist in compounds with atypical structural features which may result in non-superimposable mirror images, such as molecules with twisted structures or with hindered rotation.

Enantiomers of a given chiral compound have identical physical properties and have identical chemical properties when reacted with non-chiral

reagents. Enantiomers can be distinguished by the fact that equimolar quantities of the two enantiomers will rotate the plane of polarized light by equal amounts in the opposite direction. As well, enantiomers can also be differentiated in that they may react differently with a chiral reagent. This reaction difference may result in different reaction rates for each enantiomer and/or differences in the product's stereochemistry.

The chromatographic separation of optical isomers has been studied for many years [1,2]. An enantiomeric pair will interact in the same manner with traditional, non-chiral liquid chromatographic stationary phases. A chiral environment is needed for their separation and analysis. As was mentioned in the introduction, there are two strategies for the direct separation of enantiomers; one is the use of chiral stationary phases (CSP) [3] while the second employs a chiral mobile phase (CMP) [4,5]. This latter approach involves the incorporation of a chiral complexing agent in the mobile phase while using an achiral stationary phase. One common type of chiral additive is cyclodextrin, whose secondary hydroxyl groups are situated at the rim of the larger end of the torus while the primary hydroxyls can be found at the edge of the smaller end [6]. In order for enantioselectivity to occur, the solute must have a hydrophobic moiety suitably sized to form an inclusion complex with the cyclodextrin cavity. Once an inclusion complex is formed, each enantiomer may interact differently with the chiral functional groups at the edges of the cavity. β-Cyclodextrin, which is comprised of seven glucose units, is the most popular of the cyclodextrins used as chiral mobile phase additives.

β-Cyclodextrin has been used in the separation of the enantiomers of barbiturates [7], dansyl amino acids [7,8], and trimeprazine [9]. The main drawback of β-cyclodextrin as a chiral mobile phase additive is its limited water solubility [10]. Modification of the hydroxyl groups of the cyclodextrin alters its solubility characteristics and increases the cavity hydrophobicity.

This increase in hydrophobicity allows for the formation of stronger inclusion complexes [6] and in some cases may increase the water solubility. Modified β-cyclodextrins have been used in the separation of propranolol [11], barbiturates [12], benzoin [13], and amino acids as their dansyl and (S)-1-(1-naphthyl) ethyl isocanate derivatives [14].

Amino acid enantiomers cannot be directly separated using β-cyclodextrin phases since they are too small to form inclusion complexes. Instead, they must first be derivatized with an aromatic moiety which will form the inclusion complex allowing the chiral part to interact with the rim functional groups.

The work presented in this chapter describes the separation of amino acid enantiomers derivatized as their N-3,5-dinitrobenzoyl (DNB) and N-2,4-dinitrophenyl (DNP) derivatives with heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD) on a C_{18} column. It will be shown that the derivatized cyclodextrin can act as a better chiral selector than the underivatized one. As well, the separation of DNB-methylbenzylamine enantiomers will be described.

3.2 EXPERIMENTAL

3.2.1 Apparatus

Chromatography was performed using a liquid chromatographic system consisting of a Model M6000-A pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injector containing a 10 µl loop (Rheodyne, Cotati, CA, U.S.A.), and a Model LC290 UV detector (Perkin Elmer, Norwalk, CN, U.S.A.). The chromatograms were recorded on a Model DE120 strip chart recorder (Goerz Electro, Austria). The column was a 5 µm Zorbax ODS (250 x 4.6 mm I.D.), purchased from Chromatographic Specialties (Brockville, ON, Canada). When not in use, the column was stored in 100% methanol.

3.2.2 Chemicals

DNP-DL-methionine and DNP-DL-ethionine were obtained from Sigma (St. Louis, MO, U.S.A.). DNB-DL-leucine, DNB-DL-phenylglycine, DNB-DL-α-methylbenzylamine β-cyclodextrin, heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and triacetyl-β-cyclodextrin (TA-β-CD) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade methanol, acetonitrile, and tetrahydrofuran was purchased from Fisher (Fair Lawn, NJ, U.S.A.). Monobasic potassium phosphate, dibasic potassium phosphate, and phosphoric acid were obtained from A&C Chemicals (Montreal, QC, Canada). Water was doubly distilled and deionized.

3.2.3 Procedures

The mobile phase was prepared by mixing methanol with potassium phosphate buffer. Cyclodextrin was dissolved and the mixture was degassed and filtered through a 0.45 μm membrane filter. The pH was lowered with 10% phosphoric acid and raised with dibasic potassium phosphate. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 1 μl. The flow-rate was 0.6 ml/min. The wavelength of detection was 254 nm.

Retention times were determined by averaging at least three separate determinations. A reproducibility study was conducted where five injections had an RSD of less than 2% for the capacity factor and of less than 7% for the resolution factor.

The solubility of the cyclodextrins was determined by preparing a saturated solution of the appropriate cyclodextrin in a small amount of the mobile phase. To ensure complete saturation, the solution was heated to 75°C, shaken, sonicated, and the undissolved cyclodextrin was allowed to settle overnight. The solution was filtered and a 5 ml aliquot of the filtrate was

evaporated at 75°C. The residue was cooled to constant weight. Each determination was performed in triplicate.

3.3 DISCUSSION

3.3.1 Solubility of β-Cyclodextrins

As was mentioned in an earlier chapter, the derivatization of the rim functional groups of β-cyclodextrin often increased its water solubility. This is an important consideration as the main disadvantage of using β-cyclodextrin as a mobile phase additive is its limited water solubility. The solubilities of β-cyclodextrin, dimethyl-β-cyclodextrin, and triacetyl-β-cyclodextrin were determined to find a suitable cyclodextrin derivative. Table 3.1 presents the solubility of these three cyclodextrins at various water to methanol concentrations. The relative standard deviation, in parentheses, was for three determinations.

TABLE 3.1
Solubility (g/100 ml) of β-CD, DM-β-CD, and TA-β-CD

% Methanol	β-CD	DM-β-CD	TA-β-CD
0	1.79 (.712%)	4.01 (1.23%)	Not soluble
25	0.87 (3.20%)	4.85 (2.34%)	0.02 (25.1%)
50	0.49 (5.64%)	6.34 (4.56%)	0.03 (22.3%)
75	0.17 (2.24%)	6.89 (3.78%)	0.47 (1.50%)
100	0.04 (3.77%)	7.24 (3.25%)	0.51 (1.20%)

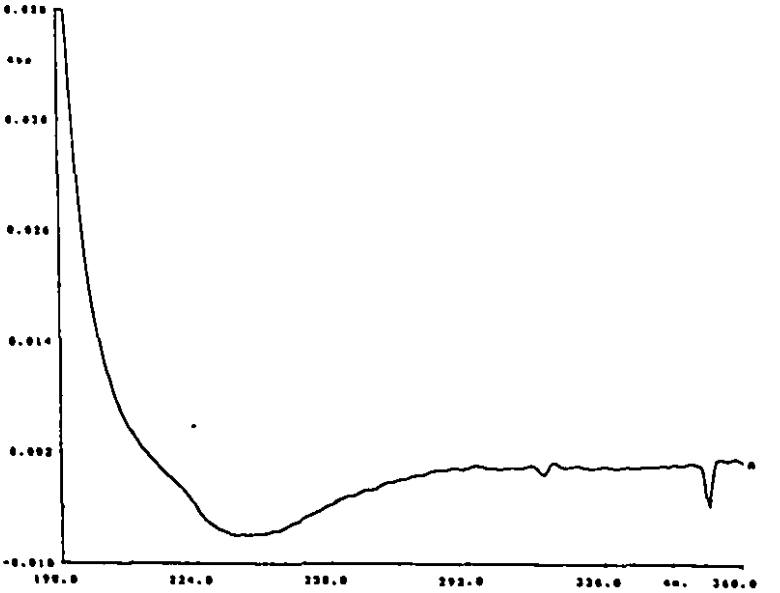
It is clear that, due to its low water solubility, triacetyl-β-cyclodextrin is unsuitable as a mobile phase additive. The high relative standard deviations was a result of the high variability of the low solubility at low methanol concentrations. The dimethyl-β-cyclodextrin is more soluble than β-cyclodextrin making it more suitable than the underivatized β-cyclodextrin as a mobile phase additive. The solubility of underivatized β-cyclodextrin corresponds to results published by Taghvaei and Stewart [10], who studied the effect of various organic mobile phase modifiers on the solubility of underivatized β-cyclodextrin.

3.3.2 Comparison of β-Cyclodextrin and DM-β-Cyclodextrin

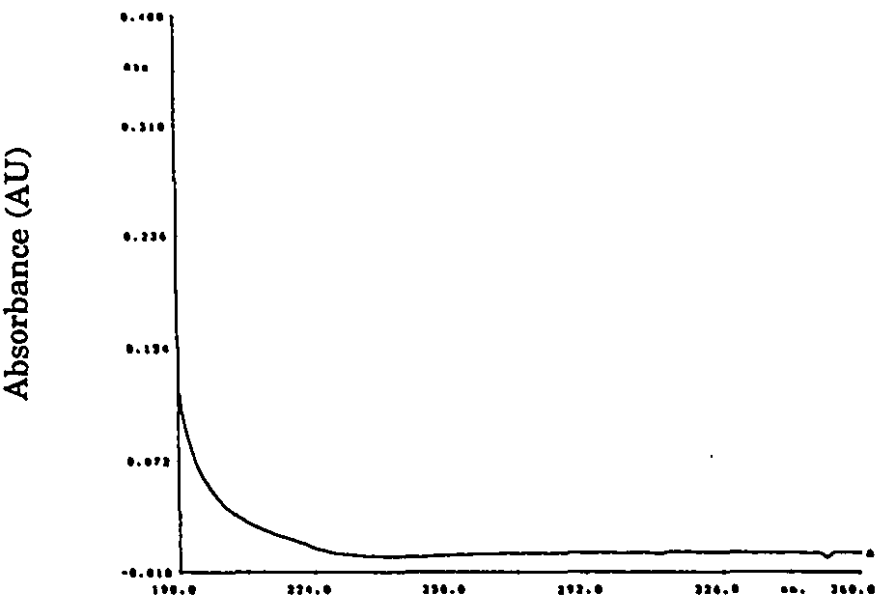
In this investigation, the chromatographic separation of four derivatized amino acid enantiomers was examined using DM-β-cyclodextrin as a chiral mobile phase additive with a non-chiral C_{18} column. Figure 3.1 shows the U.V. spectra for β-cyclodextrin and DM-β-cyclodextrin. Neither compound has appreciable absorbance between 200-350 nm, the wavelengths used most often in HPLC therefore both cyclodextrins are suitable for use as mobile phase additives.

Heptakis(2,6-di-O-methyl)-β-cyclodextrin was successful in the chiral separation of some dinitrophenyl and dinitrobenzoyl amino acids. Figure 3.2 shows the separation of all four amino acid derivatives under consideration.

The stationary phase chosen was a bonded octadecylsilane (C_{18}) column as this provided the best conditions for enantioselectivity. Two other stationary phases were examined: a phenyl and a poly(styrene-divenyl benzene) (PRP-1) column. The phenyl column did not retain the derivatized amino acids to any degree, even at very low methanol mobile phase concentrations. The polymer PRP-1 column retained the derivatized amino acids too long to be of any utility.



A



B

Figure 3.1 U.V. Spectra of (A) β-cyclodextrin and (B) DM-β-CD.

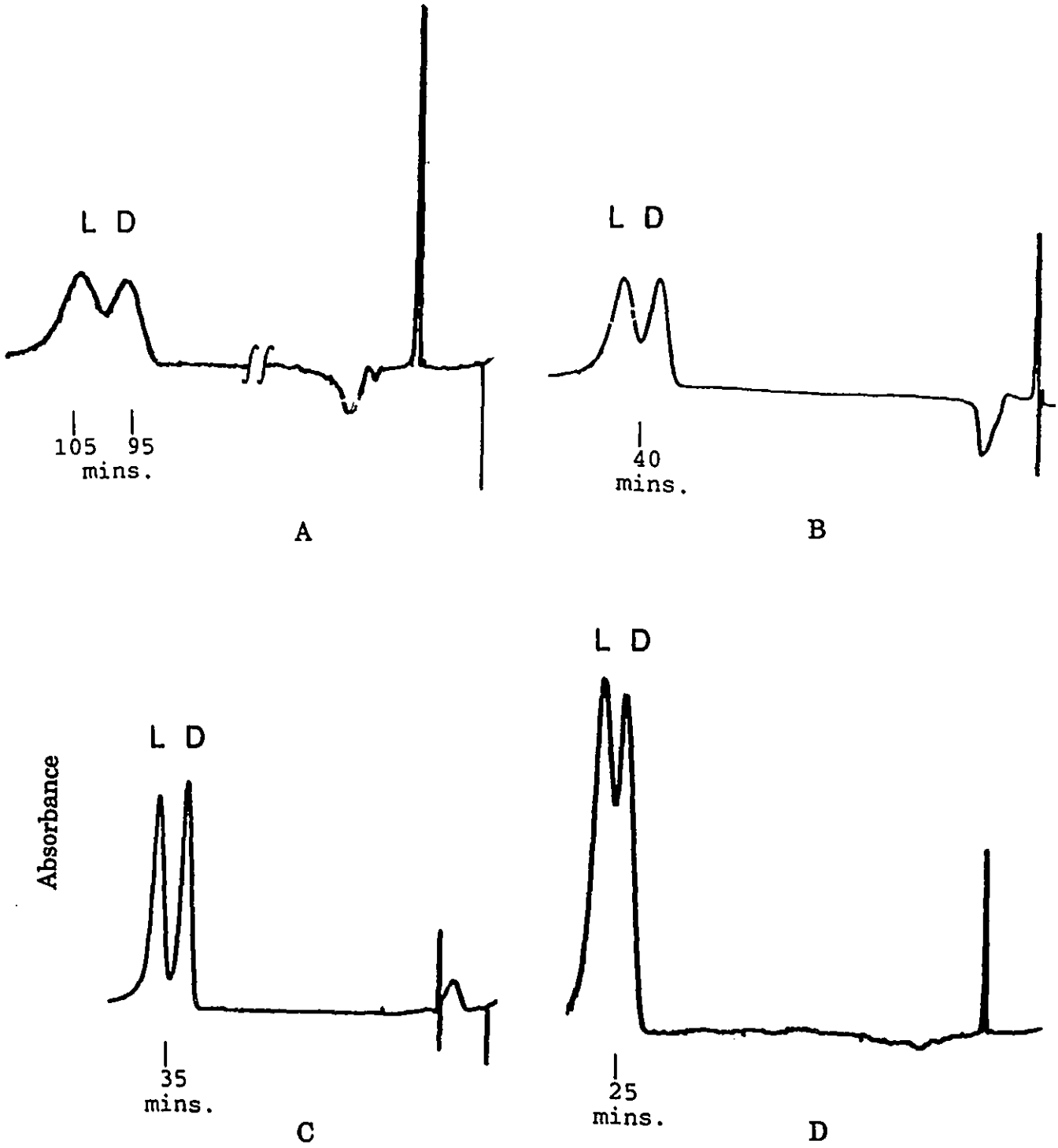


Figure 3.2 Separation of derivatized amino acid enantiomers. (A) DNB-LEU; (B) DNB-PhGLY; (C) DNP-MET; (D) DNP-ETH. Mobile Phase: 60:40 0.05M KH_2PO_4:methanol, 7.5 mg/ml DM-β-CD.

The mobile phase organic modifier chosen for this investigation was methanol. Preliminary studies showed that acetonitrile/water and tetrahydrofuran/water mobile phase mixtures resulted in significant reduction or loss of enantioselectivity. Acetonitrile and THF are stronger solvents than methanol and therefore inhibit inclusion complex formation and reduce enantioselectivity to a greater degree than methanol. Figure 3.3 shows that DNP-LEU enantiomers could not be separated with these organic modifiers.

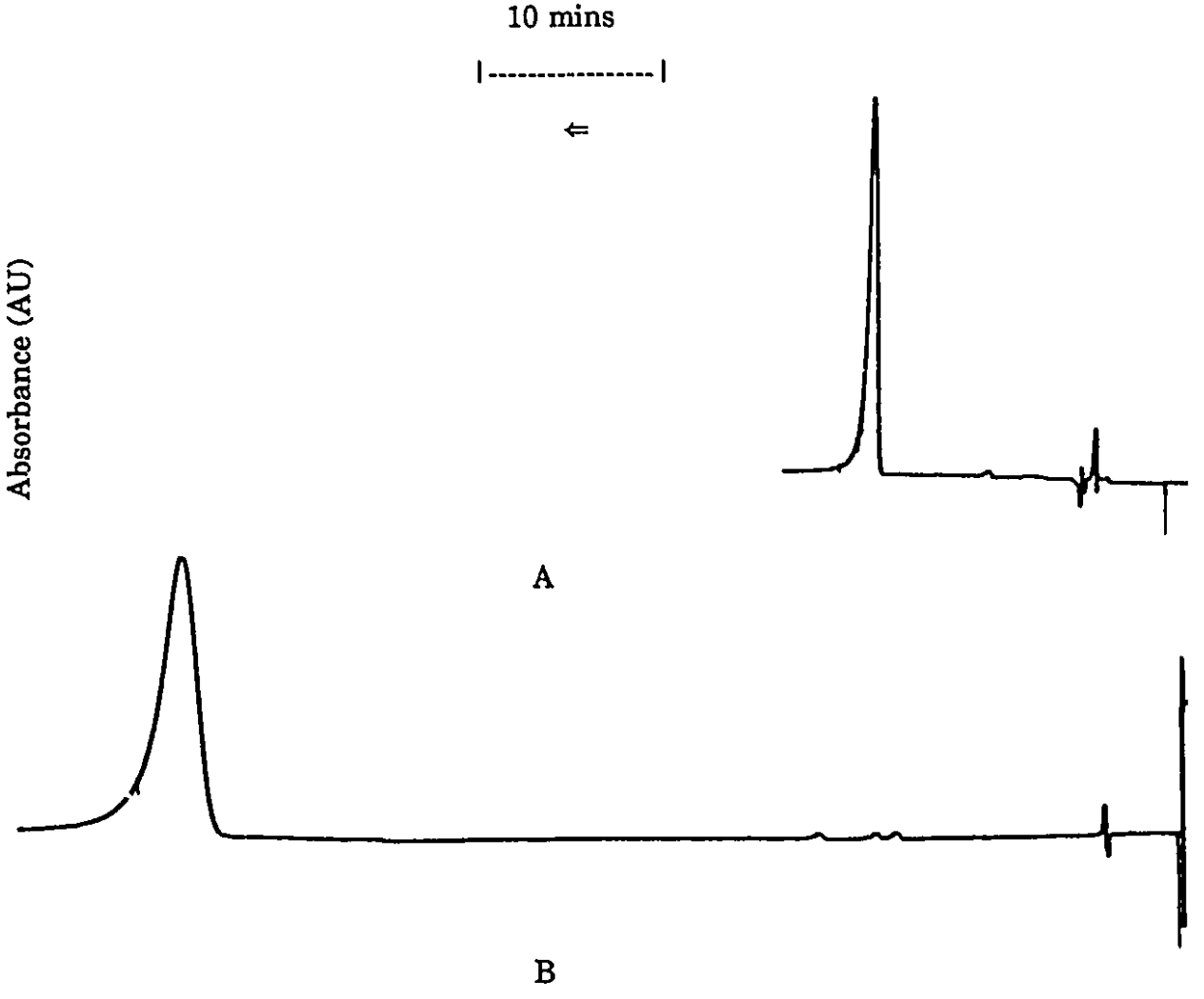


Figure 3.3 DNP-leucine enantiomers with other organic modifiers; (A) 70:30 0.05M KH_2PO_4:CH_3CN (B) 75:25 KH_2PO_4:THF; 7.5 mg/ml DM-β-CD in each mobile phase.

Table 3.2 compares the separation obtained when the chiral mobile phase additive was β-cyclodextrin with when it was DM-β-cyclodextrin. This comparison was performed with mobile phases containing two different methanol concentrations. The retention times, and therefore the capacity factors, of the amino acids were quite high but this was necessary as the solutes had to remain in the column an extended period of time to allow for adequate enantioselectivity. The use of a shorter column (15 cm in length) did not result in appreciable enantioselectivity.

In the case of the DNB-amino acids, there was a significant increase in enantioselectivity when the modified β-cyclodextrin was the chiral mobile phase additive over the unmodified β-cyclodextrin. This trend was observed at both methanol concentrations. The increase in cavity hydrophobicity due to modification of the secondary hydroxyl groups results in an increase in inclusion complex strength which allows for greater enantioselectivity. For the DNP-amino acids, there was modest separation with β-cyclodextrin in the mobile phase. There was an increase in resolution and separation factor at 40% methanol when going to the derivatized β-cyclodextrin but at 30% methanol, there was no appreciable change in separation factor or resolution factor. The increase in mobile phase polarity increased the solute fraction in the stationary phase resulting in reduced time for inclusion complex formation.

TABLE 3.2

Separations Comparing Methyl and Unmodified β-Cyclodextrin

MOBILE PHASE	Unmodified β-CD		DM-β-CD	
60:40 0.05M KH_2PO_4:MeOH	α	R_s	α	R_s
DNB-LEU	1.00	0.00	1.06	1.20
DNB-PhGly	1.00	0.00	1.08	1.10
DNP-MET	1.04	0.95	1.06	1.35
DNP-ETH	1.04	0.79	1.05	1.24
70:30 0.05M KH_2PO_4:MeOH				
DNB-LEU	1.02	0.71	1.11	1.36
DNB-PhGly	1.03	0.81	1.11	0.93
DNP-MET	1.07	1.12	1.06	1.21
DNP-ETH	1.08	1.23	1.08	1.25

* All mobile phases contained 7.5 mg/ml of the indicated cyclodextrin

Figure 3.4 demonstrates the increase in enantioselectivity when the unmodified β-cyclodextrin chiral mobile phase additive is replaced with DM-β-cyclodextrin. The example shown here is that of DNB-phenylglycine. It is evident that the modified cyclodextrin is a better chiral selector than the underivatized one. Replacing β-cyclodextrin with DM-β-cyclodextrin results in both shorter retention times and greater enantioselectivity, an indication that the derivatized amino acid forms a stronger inclusion complex with DM-β-cyclodextrin than with β-cyclodextrin. This trend was observed with all the derivatized amino acids. These findings were the opposite of those for dansylated amino acids [14]. The authors reported that the methylation of β-cyclodextrin actually decreased the enantioselectivity of dansyl amino acids.

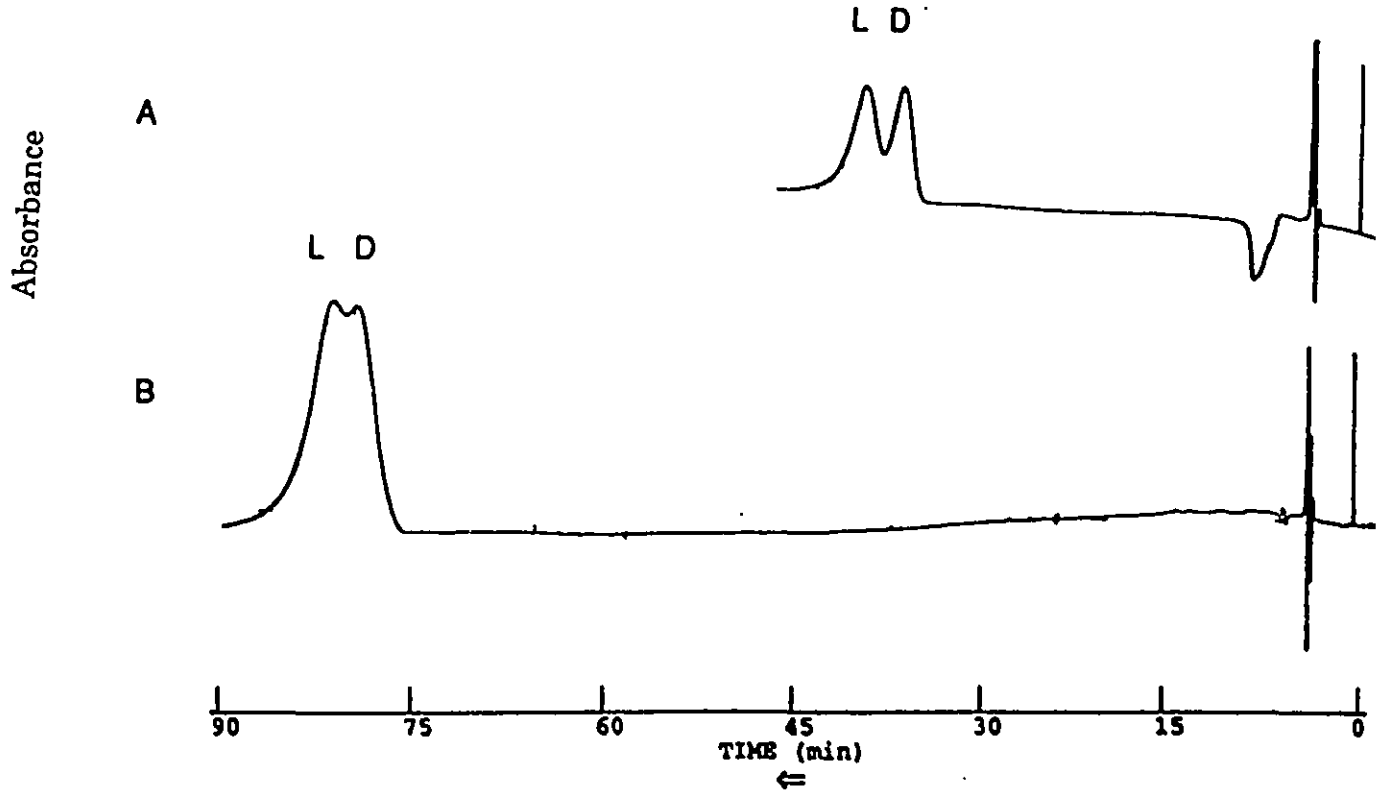


Figure 3.4 Separation of dinitrobenzoyl-phenylglycine enantiomers (A) Methyl-β-Cyclodextrin in mobile phase; (B) β-Cyclodextrin in mobile phase. Mobile phase: 70:30 0.05M KH_2PO_4:methanol with 7.5 mg/ml of the indicated additive.

3.3.3 Effect of DM-β-cyclodextrin concentration

The effect of DM-β-cyclodextrin on enantioselectivity and retention was studied by varying the cyclodextrin concentration in the mobile phase. The mobile phase composition was held constant at 60:40 0.05M KH_2PO_4:methanol. Figure 3.5 presents the effect of cyclodextrin concentration on the separation of the amino acid enantiomers.

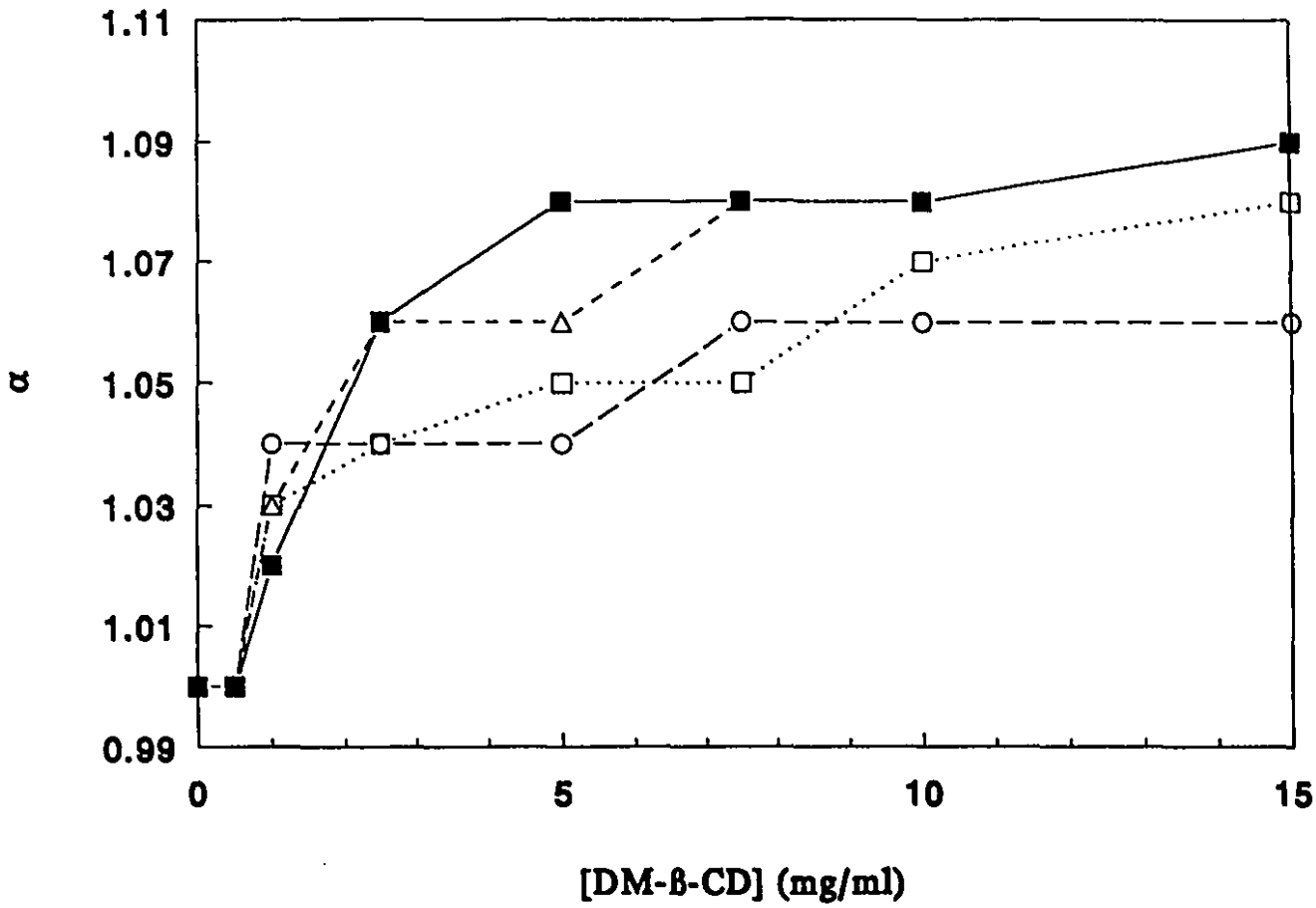


Figure 3.5 Effect of methyl-β-cyclodextrin concentration on the separation factor of different derivatized amino acids. Mobile phase is 60:40 0.05M KH_2PO_4:methanol plus indicated amount of methyl-β-CD. ■ = DNB-LEU; Δ = DNB-PhGLY; O = DNP-MET; □ = DNP-ETH.

The separation profile is characterized by no separation at low cyclodextrin concentrations, followed by a rapid increase in separation factor, and then a plateau region where large increases in cyclodextrin concentration have little effect on separation. This knowledge serves as a guide for choosing the correct cyclodextrin concentration, taking into account selectivity and concentration, as the latter may adversely affect pump component life due to high solids concentrations in the mobile phase.

As the cyclodextrin concentration was increased, the capacity factor (k') decreased significantly. Table 3.3 shows the capacity factor for the first eluting amino acid enantiomer as a function of DM-β-cyclodextrin concentration.

TABLE 3.3

Capacity Factors vs DM-β-cyclodextrin Concentration

[m-β-CD] (mg/ml)	DNB-LEU	DNB-PhGly	DNP-MET	DNP-ETH
0.0	26.5	12.4	19.3	35.6
0.5	24.9	11.1	20.8	44.3
1.0	25.6	11.8	24.0	49.0
2.5	22.1	10.7	21.8	44.0
5.0	20.2	9.87	21.5	41.2
7.5	14.9	7.44	15.2	28.7
10.0	14.9	7.44	14.9	27.8
15.0	14.0	6.81	13.1	23.4

* The mobile phase was composed of 60:40 0.05M KH_2PO_4:Methanol

The reduction in capacity factor is a result of the solute being transported down the column by the cyclodextrin. In the absence of cyclodextrins in the mobile phase, the derivatized amino acids have a high affinity to the hydrophobic C_{18} column resulting in high capacity factors.

3.3.4 Effect of Methanol Concentration

The effect of varying the methanol concentration in the mobile phase was studied by varying the methanol-phosphate buffer ratio. Figure 3.6 shows

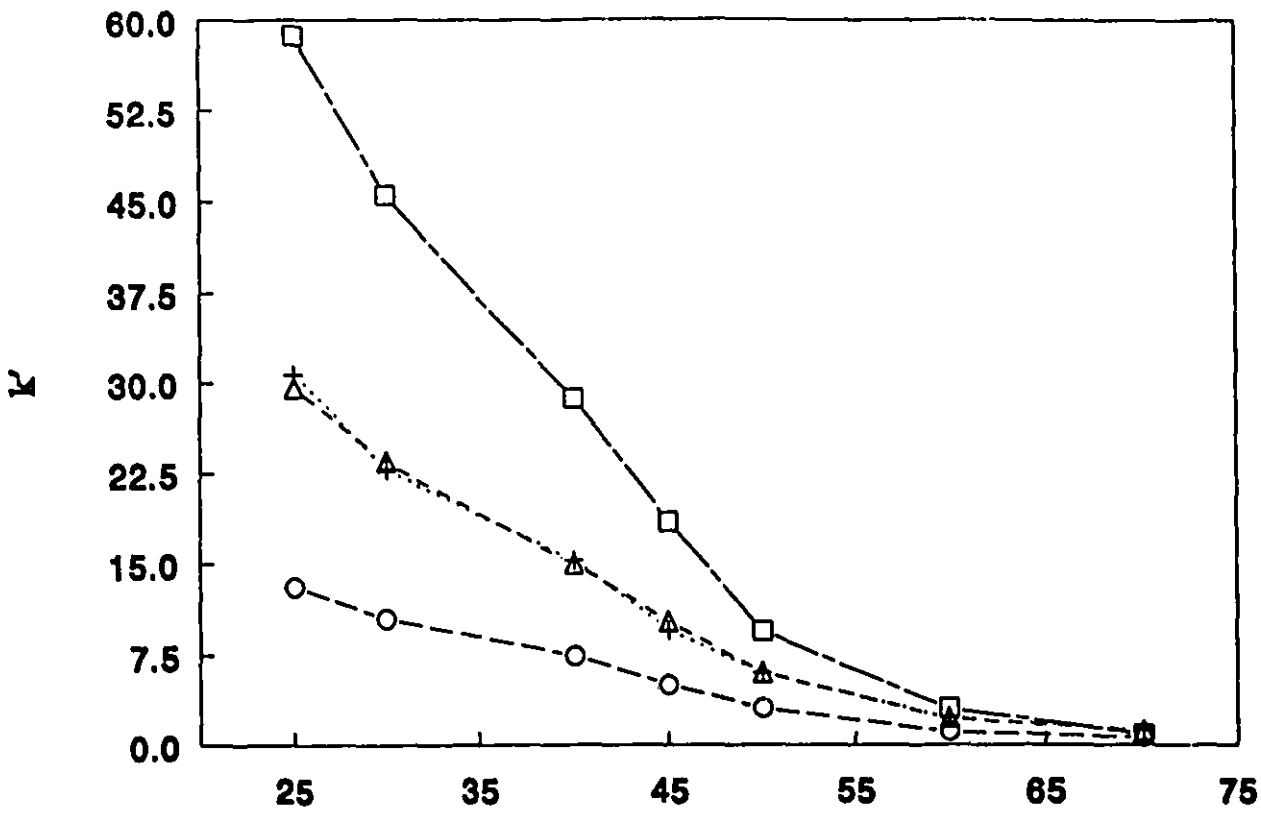


Figure 3.6 Effect of methanol concentration on the capacity factor of different derivatized amino acids. Mobile phase contains 7.5 mg/ml of methyl-β-CD. Δ = DNB-LEU; ○ = DNB-PhGLY; + = DNP-MET; □ = DNP-ETH.

the effect of methanol mobile phase concentration on the capacity factor of the DNB- and DNP-amino acids. The DM-β-CD concentration was maintained constant at 7.5 mg/ml.

It was observed that the capacity factor decreased with increasing methanol concentration. As the mobile phase becomes less polar, its solvent strength increases and the solutes have a greater affinity for the mobile phase and a shorter retention time [15]. Figure 3.7 shows the effect of methanol concentration on the separation factor of the amino acid enantiomers.

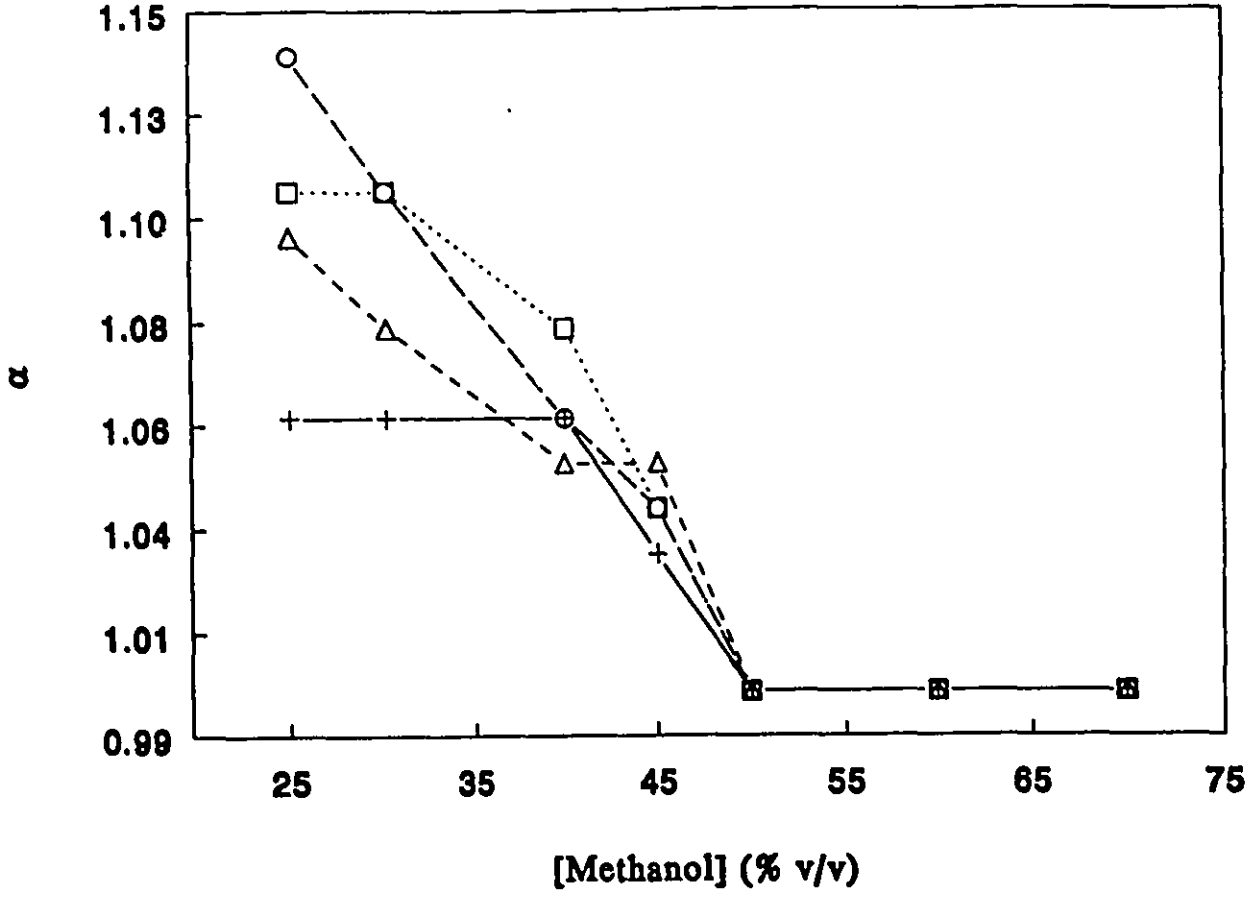


Figure 3.7 Effect of methanol concentration on the separation factor of different derivatized amino acids. Mobile phase contains 7.5 mg/ml of methyl-β-CD. O = DNB-LEU; Δ = DNB-PhGLY; + = DNP-MET; □ = DNP-ETH.

DM-β-cyclodextrin provides no enantioselectivity at high methanol concentrations as the relatively low mobile phase polarity discourages the formation of inclusion complexes necessary for enantioselectivity. Enantioselectivity increases significantly, beginning at about 50% methanol, as inclusion complex strength increases due to the increase in mobile phase polarity at the lower methanol concentrations. The trend of increasing enantioselectivity with decreasing methanol concentration has also been observed for DNP-amino acids with β-cyclodextrin stationary phases [3].

3.3.5 Effect of pH

The influence of changing the pH of the mobile phase on retention and separation was investigated. The pH of the mobile phase is an important parameter for ionizable solutes as the degree of ionization will affect their retention profile and resolution [15]. Figure 3.8 is a plot of separation factor versus the separation of the DNB-leucine, DNB-phenyl glycine, DNP-methionine, and DNP-ethionine enantiomers.

The effect of varying the mobile phase pH was studied over a range of 3 to 8 as the silica-based column was unstable outside this range. The influence of pH was not overly significant as the amino acids exist mainly in their anionic form. The pH has a greater effect on the DNB-amino acids than on the DNP-amino acids which indicates that the polar groups of the DNB-amino acids play a greater role in enantioselectivity than those of the DNP-amino acids. In all cases, there was a decrease in enantioselectivity as the pH is raised as the carboxyl groups acids become less protonated.

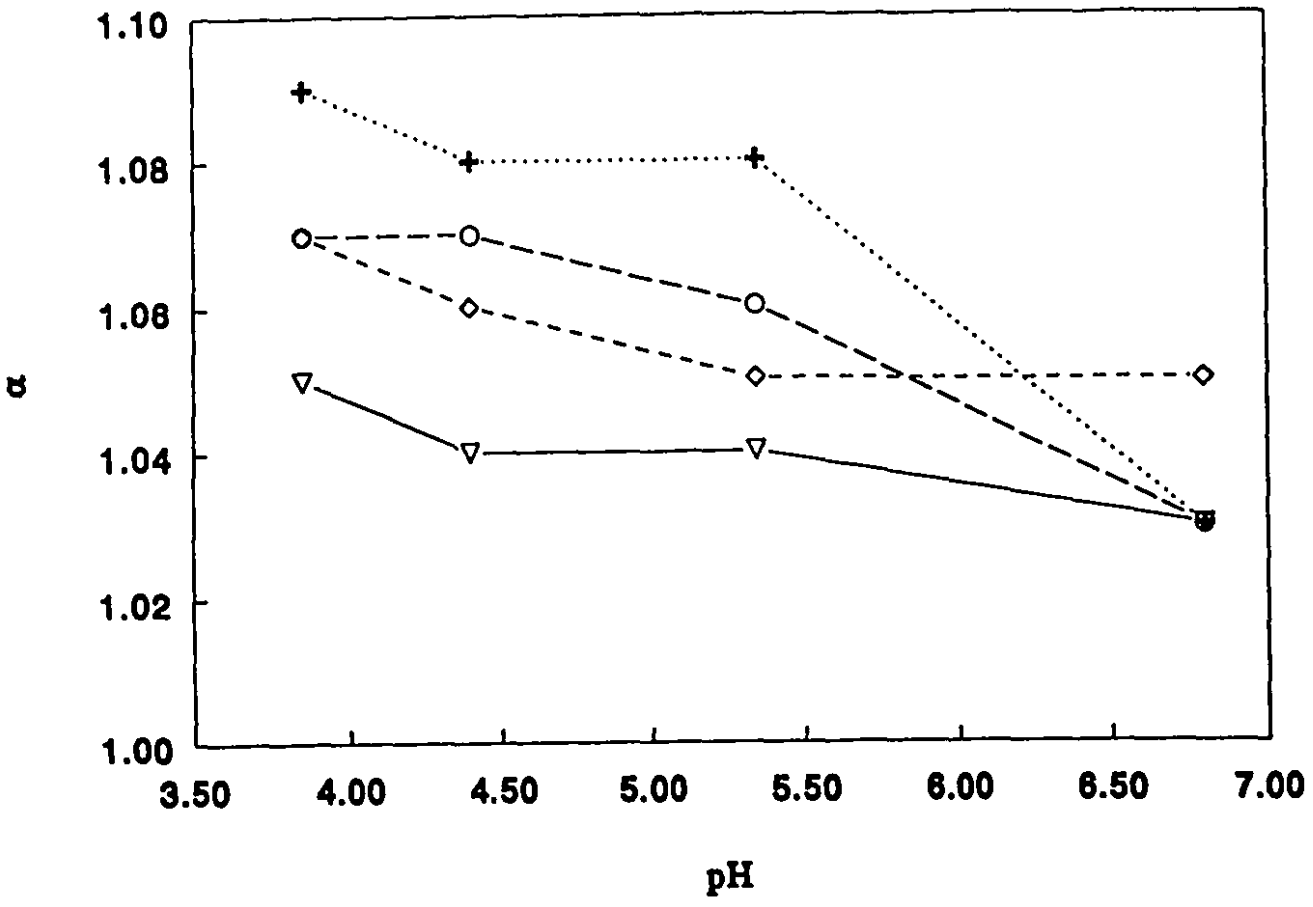


Figure 3.8 Effect of pH on the separation factor of different derivatized amino acids. Conditions: 60:40 0.05M KH_2PO_4:methanol, 7.5 mg/ml methyl-β-CD. O = DNB-LEU; + = DNB-PhGLY; ▽ = DNP-MET; ◊ = DNP-ETH.

Increasing the mobile phase pH had a much greater effect on the capacity factor, as is shown in Table 3.4. The reported capacity factor is that of the first eluting enantiomer. In all cases, there was a significant reduction in capacity factor as the pH was raised due to reduced interaction with the stationary phase.

TABLE 3.4

Effect of pH on Capacity Factor

pH	DNB-LEU	DNB-PhGly	DNP-MET	DNP-ETH
3.85	46.3	19.1	33.8	62.5
4.40	39.2	15.1	22.0	40.1
5.35	14.0	6.94	15.2	26.9
6.80	9.06	5.94	11.4	20.9

Mobile phase: 60:40 0.05M KH_2PO_4:Methanol, 7.5 mg/ml DM-β-cyclodextrin.

3.3.6 Effect of Buffer Concentration

The effect of varying the KH_2PO_4 concentration on separation and retention in the mobile phase was studied. Figure 3.9 is a plot of separation factor versus KH_2PO_4 concentration for the amino acid derivatives.

Generally, the buffer concentration had little effect on the enantioselectivity of the DNB- and DNP-amino acids. This is not surprising since KH_2PO_4 is too small to compete with the solutes for inclusion complexation. One exception was that DNP-methionine showed no enantioselectivity at a low buffer concentration (0.02M). Separation of the enantiomers peaked at a buffer concentration of 0.05M, and then levels off. Similarly, buffer concentration had little effect on capacity factor.

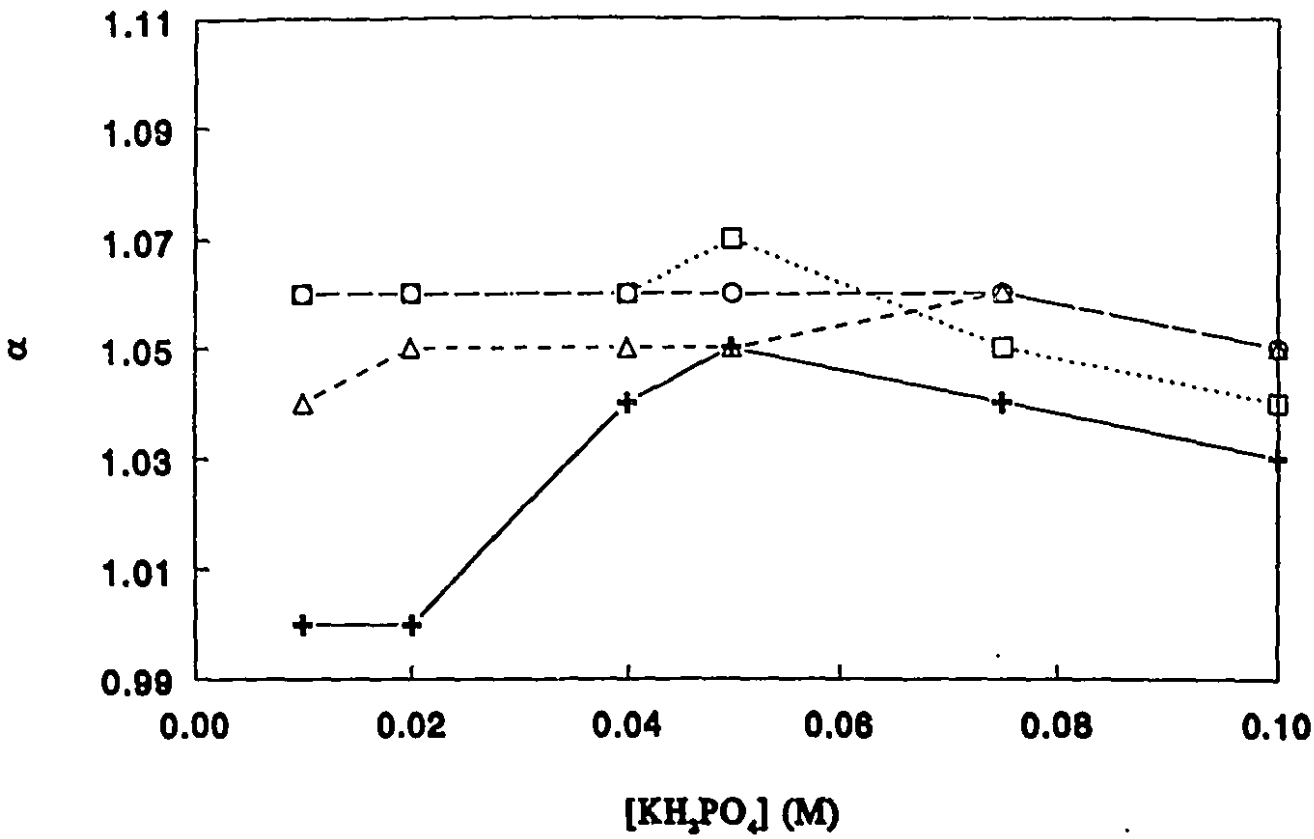


Figure 3.9 Effect of buffer concentration on the separation factor of different derivatized amino acids. Conditions: 60:40 KH_2PO_4 buffer:methanol, 7.5 mg/ml methyl-β-CD. O = DNB-LEU; □ = DNB-PhGLY; + = DNP-MET; Δ = DNP-ETH.

3.3.7 Separation of DNB-Methylbenzylamine

DM-β-cyclodextrin is also useful in the chiral separation of compounds other than amino acids derivatized with a N-3,5-dinitrobenzoyl group. Figure 3.10 shows the enantiomeric separation of DL-DNB-α-methylbenzylamine.

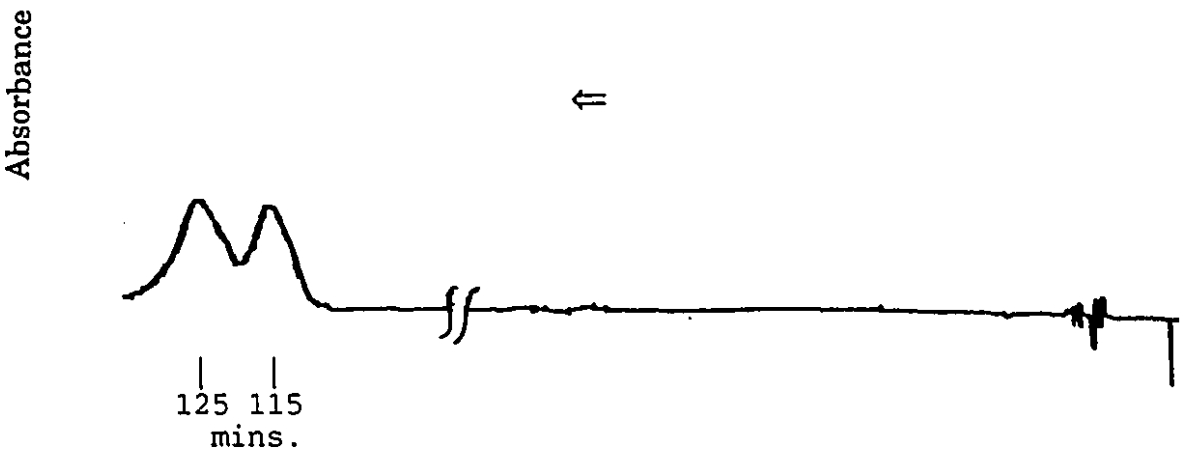


Figure 3.10 Separation of DNB-α-methylbenzylamine enantiomers. Mobile phase: 80:20 0.033M KH_2PO_4:methanol, 7.5 mg/ml DM-β-CD.

Enantioselectivity was not observed with underivatized β-cyclodextrin as the inclusion complex was not strong enough for sufficient chiral recognition. However, the increased inclusion complex strength with the DM-β-cyclodextrin allowed for chiral resolution of the DNB-methylbenzylamine enantiomers. The retention time of these enantiomers, however, was very long resulting in significant band broadening.

3.4 CONCLUSIONS

It has been demonstrated that DM-β-cyclodextrin can be used as chiral mobile phase additives in the enantiomeric separation of some dinitrobenzoyl and dinitrophenyl amino acids. It was found that there is an increase in enantioselectivity when β-cyclodextrin is derivatized as the less polar cavity forms stronger inclusion complexes with the solutes. Cyclodextrin and methanol concentration in the mobile phase, as well as its pH, have been found to be important parameters in the separation and retention of these amino acid derivatives. The use of DM-β-cyclodextrin also improves the enantioselectivity of dinitrobenzoyl-α-methylbenzylamine.

3.5 REFERENCES

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CHAPTER 4

HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF EQUILIN, ESTRONE, AND ESTRONE DERIVATIVES WITH CYCLODEXTRINS AS MOBILE PHASE ADDITIVES

4.1 INTRODUCTION

As was mentioned in chapter 2, cyclodextrins are used in high performance liquid chromatography to provide inclusion complex interactions between the cyclodextrin cavity and solute in both the stationary phase and as a mobile phase additive. They are used predominantly in the separation of optical isomers as the chiral environment at the cavity rim can be used as a chiral selector [1,2]. Cyclodextrins, however, have also been used for non-chiral applications for eluents that are similarly-structured [3]. These separations are often a challenge using conventional liquid chromatographic systems as it is difficult to distinguish between the minor differences in structure. The use of cyclodextrins can be a suitable strategy for separating these compounds providing inclusion complexation occurs. The differences in inclusion complex strengths, as well as differences in the interaction with the rim functional

groups, can improve chromatographic separation.

Steroids are generally regarded as difficult compounds to isolate and analyze [4]. Their structure and complexity of the matrix in which they are usually found make there analysis challenging. Conventional high performance liquid chromatography has been used in the analysis of steroids since the mid-1970's. Recently, however, it has been found that steroids have structural features that make them suitable for the formation of inclusion complexes with β-cyclodextrins [5-7]. Both β- and γ-cyclodextrin have been reported in their separation and analysis by high performance liquid chromatography. Shimada and Nonaka [8] separated a series of corticoids with β- and γ-cyclodextrin in the mobile phase while Lamparczyk and co-workers [9] used β-cyclodextrin as a mobile phase additive in the separation of estriol, estradiol, and estrone in human urine. Agnus and co-workers [10] separated pregnanolone and progesterone with β-cyclodextrin as a mobile phase additive while Higashidate and co-workers [11] resolved bile acids with heptakis(2,6-di-O-methyl)-β-cyclodextrin in the mobile phase.

This chapter focuses on the use of β-cyclodextrin and modified β-cyclodextrins as mobile phase additives in the separation of various steroid molecules. The modified cyclodextrins consist of having the hydroxyl groups on the rim of the cyclodextrin cavity replaced with either methyl or hydroxyethyl groups. As was mentioned previously, these modifications increase the hydrophobic character of the cyclodextrin cavity relative to the hydrophillic exterior and alter the interactions between the solute and rim groups. These differences will change the inclusion complex strength which can lead to greater selectivity.

This chapter describes and characterizes the separation of equilin and estrone, two very similarly structured steroids of importance to the pharmaceutical industry in the estrogenic hormone replacement treatment of

menopausal women. Typically, equilin and estrone are analyzed by either packed-column gas chromatography preceded by an enzymatic derivatization [12] or by capillary gas chromatography [13]. The direct analysis of equilin and estrone by HPLC without cyclodextrin has been reported [14]; however the use of cyclodextrins as a mobile phase additive greatly improves the resolution of these two molecules. To date there has been no report on the use of cyclodextrins in the separation of equilin and estrone. Also described in this chapter is the separation of estrone from three of its related derivatives/metabolites, specifically 16α-hydroxyestrone (16α-HE), 2-hydroxyestrone (2-HE), and 4-hydroxyestrone (4-HE).

A comparison of the separation obtained for these steroids with unmodified β-cyclodextrin, methyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin as mobile phase additives will be discussed. Also discussed are the effect varying mobile phase parameters such as cyclodextrin, methanol, and buffer concentration, as well as pH.

4.2 EXPERIMENTAL

4.2.1 Apparatus

The high performance liquid chromatography apparatus was described in section 3.2.1. The column used with the mobile phase additives was a 5 µm Zorbax ODS (150 x 4.6mm I.D.), purchased from Chromatographic Specialties (Brockville, ON, Canada). The β-cyclodextrin column was a Cyclobond I (250 x 4.6 mm I.D.), purchased from Advanced Separation Technologies (Whippany, NJ, U.S.A.). When not in use, the columns were stored in 100% methanol.

4.2.2 Chemicals

Equilin, estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone were obtained from Sigma (St. Louis, MO, U.S.A.). β-Cyclodex-

trin (β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and hydroxyethyl-β-cyclodextrin (HE-β-CD) were obtained from Aldrich (Milwaukee, WI, U.S.A.). HPLC-grade methanol was purchased from Fisher (Fair Lawn, NJ, U.S.A.). Monobasic potassium phosphate, dibasic potassium phosphate, and phosphoric acid were obtained from A&C Chemicals (Montreal, QC, Canada). Water was doubly distilled and deionized.

4.2.3 Procedures

The mobile phase was prepared by mixing methanol with potassium phosphate buffer. Cyclodextrin was dissolved and the mixture was degassed and filtered through a 0.45 μm membrane filter. The pH was lowered with 10% phosphoric acid and raised with dibasic potassium phosphate. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 2 μl. The wavelength of detection was 200 nm.

Retention times were determined by averaging at least three separate determinations. A reproducibility study was conducted where six injections had an RSD of less than 1% for the capacity factor and of less than 2.5% for the resolution factor.

4.3 RESULTS AND DISCUSSION

In this investigation, the chromatographic separation of equilin and estrone, as well as estrone from three of its metabolites/derivatives, was examined using β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin as mobile phase additives. Figure 4.1 presents the five molecules under investigation.

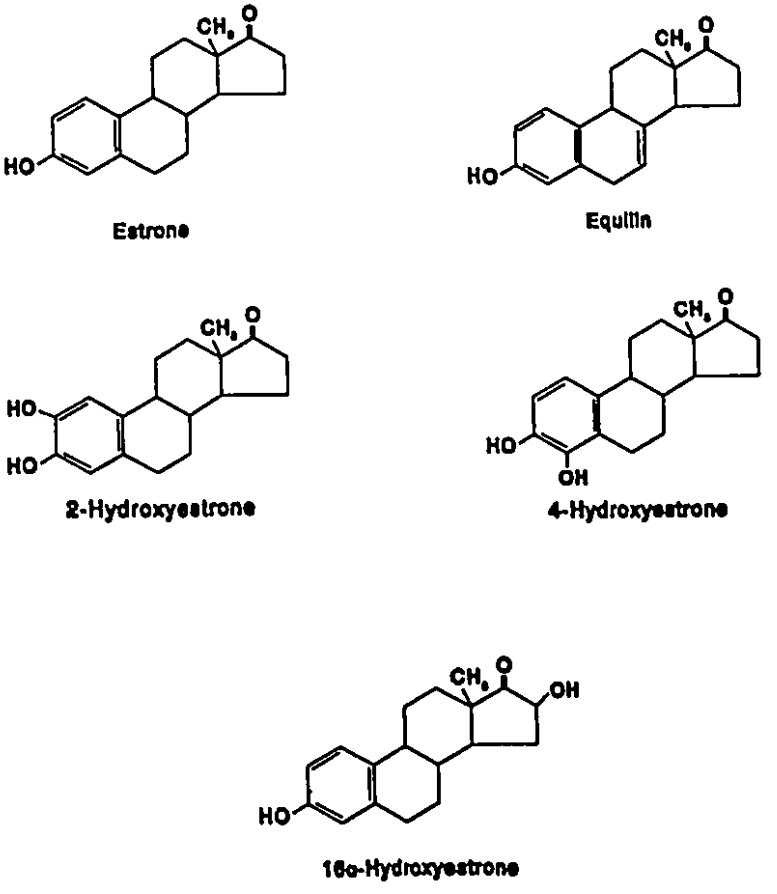

Figure 4.1 Steroids studied

The stationary phase chosen for this work was a 15 cm bonded C_{18} (octadecylsilane) column. It proved to be the most suitable for the steroid separations. Three other stationary phases were investigated: cyanopropyl (CN), aminopropyl (NH_2), and poly(styrenedivenyl benzene) (PRP-1) columns. These columns were not suitable for this analysis. The first two columns did not retain the steroids, even at low methanol concentrations, making them unsuitable for their analysis. On the other hand, the polymeric PRP-1 column retained the steroids very strongly and they did not elute in a reasonable amount of time (i.e. more than 2 hours). Their chromatograms can be seen in Figure 4.2. The chromatograms on a C_{18} column will be presented later.

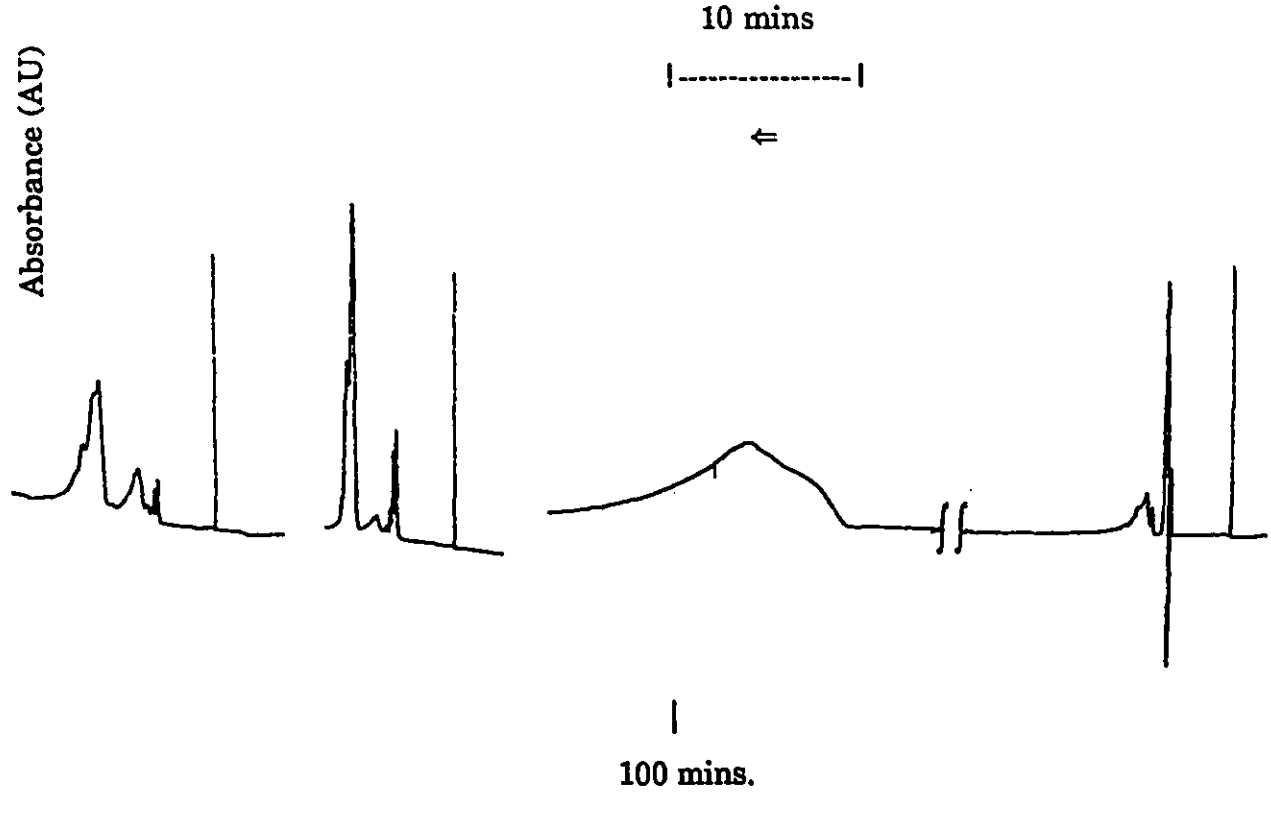

Figure 4.2 Comparison of various stationary phases in the separation of equilin and estrone. (A) CN; (B) NH_2; (C) PRP-1.

4.3.1 Separation of Equilin and Estrone

In the absence of cyclodextrins or other additives in the mobile phase, the resolution of equilin and estrone is poor. Band broadening was significant resulting in wide peaks with no baseline separation. However, the presence of cyclodextrins in the mobile phase improves the separation of the two estrogens. Figure 4.3 compares the separation of equilin and estrone when the mobile phase additive was β-cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin.

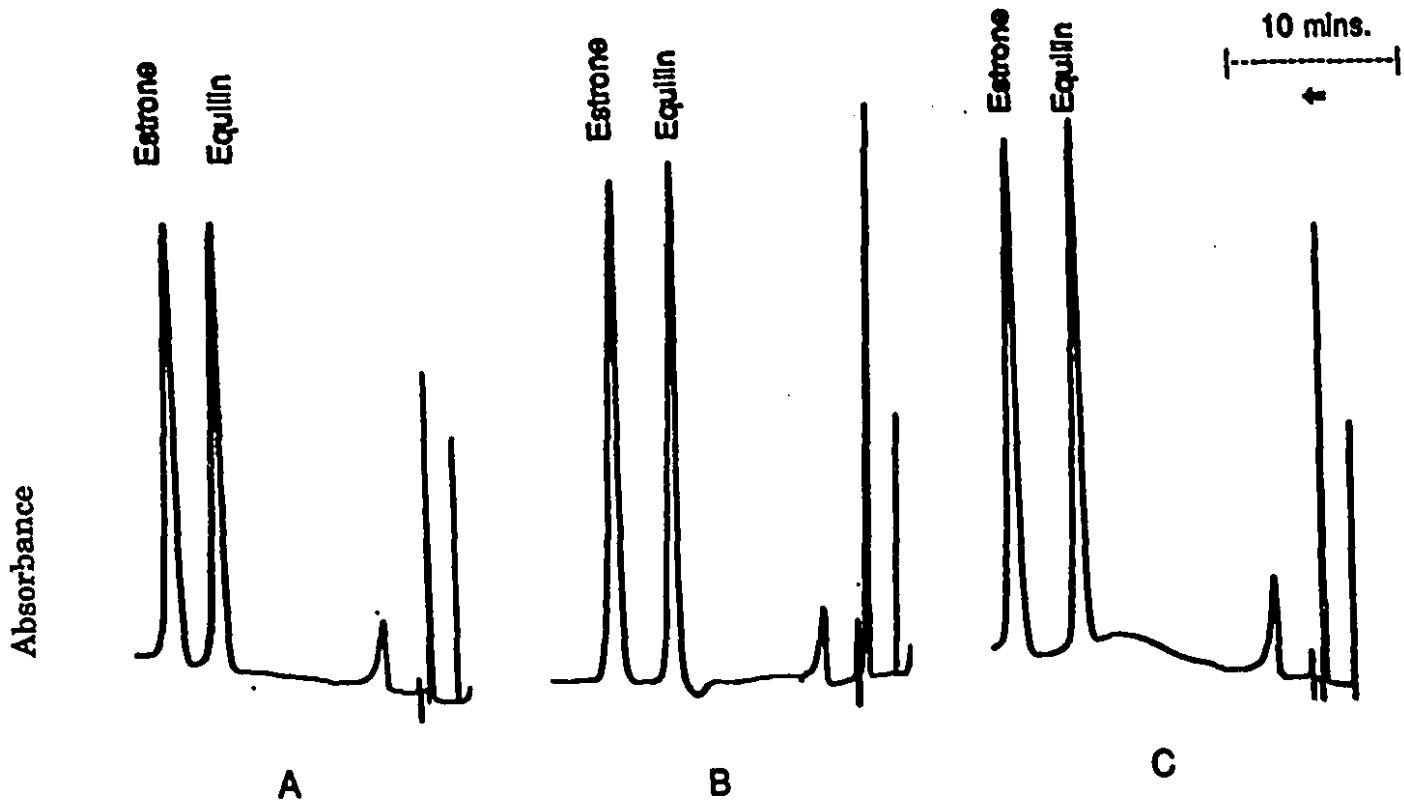

Figure 4.3 Comparison of various cyclodextrin types in separation of equilin and estrone. Mobile phase is 45:55 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) β-CD; (B) DM-β-CD; (C) HE-β-CD.

β-Cyclodextrin provides baseline separation of the two steroids. Band broadening was greatly reduced by the increase in efficiency provided by the cyclodextrin. However, the resolution is improved even further when DM- or HE-β-cyclodextrin is the mobile phase additive. Evidently, the inclusion complex strength of equilin over estrone was changed significantly enough by the derivatization of the β-cyclodextrin to result in a significant improvement in resolution.

Retention times decrease when some form of cyclodextrin is present in the mobile phase, evidence that inclusion complexes are formed. Table 4.1 summarizes the separation and retention of equilin and estrone with the various cyclodextrins in the mobile phase. These separations are compared with separations on a β-cyclodextrin stationary phase using comparable mobile phase organic modifier compositions. Similar separation was achieved using a β-cyclodextrin column, however the solutes had very short retention times. The steroids are retained a lot longer on the hydrophobic C_{18} column than on the β-cyclodextrin column. The steroids' affinity for the β-cyclodextrin stationary phase is not as high as it is for C_{18} so the capacity factors will be much higher on a C_{18} column. Addition of the cyclodextrin to the mobile phase cyclodextrin assists in eluting a solute that is strongly retained on the C_{18} stationary phase.

The β-cyclodextrin stationary phase gave reasonable separation of equilin and estrone despite the short retention times. Attempts to increase retention by increasing the aqueous ratio in the mobile phase resulted in significant band broadening and reduction in resolution. The elution order of equilin and estrone on the β-cyclodextrin is reversed when comparing the cyclodextrin mobile phase to a cyclodextrin stationary phase, further evidence of the formation of inclusion complexes.

TABLE 4.1

Retention and Separation of Equilin and Estrone Comparing Different Cyclodextrins

Mobile Phase Additive	$k'_{EQUILIN}$	$k'_{ESTRONE}$	α
No Cyclodextrin	15.8	17.6	1.11
β-Cyclodextrin	9.09	10.9	1.20
DM-β-Cyclodextrin	8.43	10.9	1.29
HE-β-Cyclodextrin	10.6	13.5	1.27
β-Cyclodextrin Stat. Phase	2.33	1.93	1.21

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin. In the case of the β-cyclodextrin stationary phase, the mobile phase is 60:40 0.05M KH_2PO_4:methanol.

4.3.2 Separation of Estrone and its Derivatives

Cyclodextrins as a mobile phase additives were also successful in separating estrone from some of its derivatives. Figure 4.4 shows an example of how the various forms β-cyclodextrin affect the resolution of estrone, 2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone.

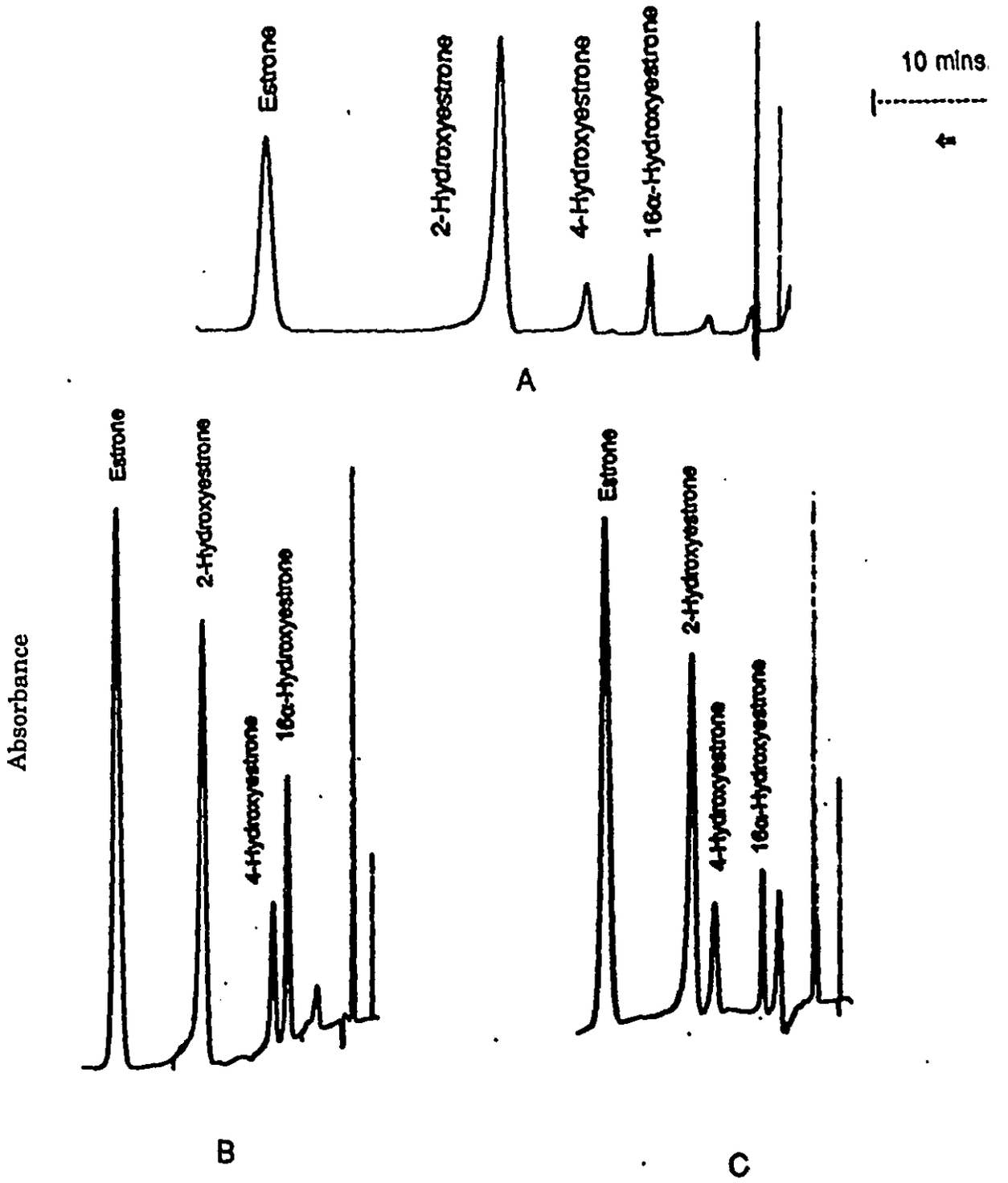

Figure 4.4 Comparison of cyclodextrin type in the mobile phase on separation of estrone from its derivatives. Mobile phase is 55:45 0.05M KH_2PO_4:methanol plus 5 mg/ml of indicated cyclodextrin. (A) HE-β-CD; (B) β-CD; (C) DM-β-CD.

The unmodified β-cyclodextrin provides suitable separation of the 2- and 4-hydroxyestrone isomers however there is barely baseline separation of the 4- and 16α-isomers. Replacing β-cyclodextrin with DM-β-cyclodextrin allows for better separation of the 4- and 16α-isomers but does not separate the 2- & 4-isomers as well as the unmodified β-cyclodextrin. The greatest separation of all four steroids occurred when the mobile phase additive was HE-β-cyclodextrin. Estrone is well separated from its hydroxy derivatives regardless of which cyclodextrin is in the mobile phase. Table 4.2 shows the relationship between capacity factor for estrone and its three derivatives and the type of cyclodextrin used in the mobile phase. Table 4.3 lists how each type of cyclodextrin affects the separation factors for the similarly structured 2- and 4-hydroxyestrones and the 4- and 16α-hydroxyestrones. In the latter case, the addition of cyclodextrin actually decreased the separation factor though it greatly reduced the capacity factors. The mobile phases for the data in tables 4.2 and 4.3 are 55:45 0.05M KH_2PO_4:methanol with 5 mg/ml of the indicated cyclodextrin. As was noted in Figure 4.4, β-cyclodextrin provides the best separation of the 2- and 4-isomers. As was the case with equilin and estrone, the significant decreases in capacity factor for all the steroids indicate that they did indeed form inclusion complexes with all the cyclodextrins studied here.

TABLE 4.2

Comparison of Capacity Factors for Estrone and its Derivatives

Mobile Phase Additive	$k'_{ESTRONE}$	$k'_{2\text{-HE}}$	$k'_{4\text{-HE}}$	$k'_{16\alpha\text{-HE}}$
No Cyclodextrin	88.8	44.5	45.5	17.2
β-Cyclodextrin	16.3	10.3	5.49	4.63
DM-β-CD	13.0	7.92	6.63	3.92
HE-β-CD	29.4	15.6	10.6	6.92

TABLE 4.3

Comparison of Separation Factors for Estrone's Derivatives

Mobile Phase Additive	$\alpha_{2\text{-}\&4\text{-HE}}$	$\alpha_{4\text{-}\&16\alpha\text{-HE}}$
No Cyclodextrin	1.02	2.64
β-Cyclodextrin	1.88	1.19
DM-β-CD	1.19	1.69
HE-β-CD	1.47	1.53

4.3.3 Effect of Cyclodextrin Concentration

The effect of varying the cyclodextrin in the mobile phase was examined for the separation of equilin and estrone as well as for estrone and its derivatives. For the first part, only the effect of heptakis(2,6-di-O-methyl)-β-cyclodextrin concentration was investigated. The chromatograms shown in figure 4.5 presents the effect of mobile phase DM-β-cyclodextrin concentration on the separation of equilin and estrone. With no cyclodextrin in the mobile phase, the peaks are poorly shaped and exhibit considerable band broadening. The addition of even a small amount of DM-β-cyclodextrin to the mobile phase resulted in a significant increase in resolution. As the cyclodextrin concentration is increased, the capacity factors for equilin and estrone decrease dramatically indicating the formation of relatively strong inclusion complexes. The reduction in capacity factor is accompanied by an increase in separation factor.

The best separation was obtained at the highest cyclodextrin concentration (13 mg/ml) examined here, however the high concentration of the additive to the mobile phase had adverse effects on the solvent delivery pump. The cyclodextrin had a tendency of precipitating out on various components such as the draw-off valve, the plunger, and the inlet and outlet valves and seals. As well, the cyclodextrin would precipitate out in the rotor valve and sample loop on the injector. For practical purposes, extremely high cyclodextrin concentrations should be avoided in favour of more compatible concentrations (e.g. 5 mg/ml) to the chromatographic system.

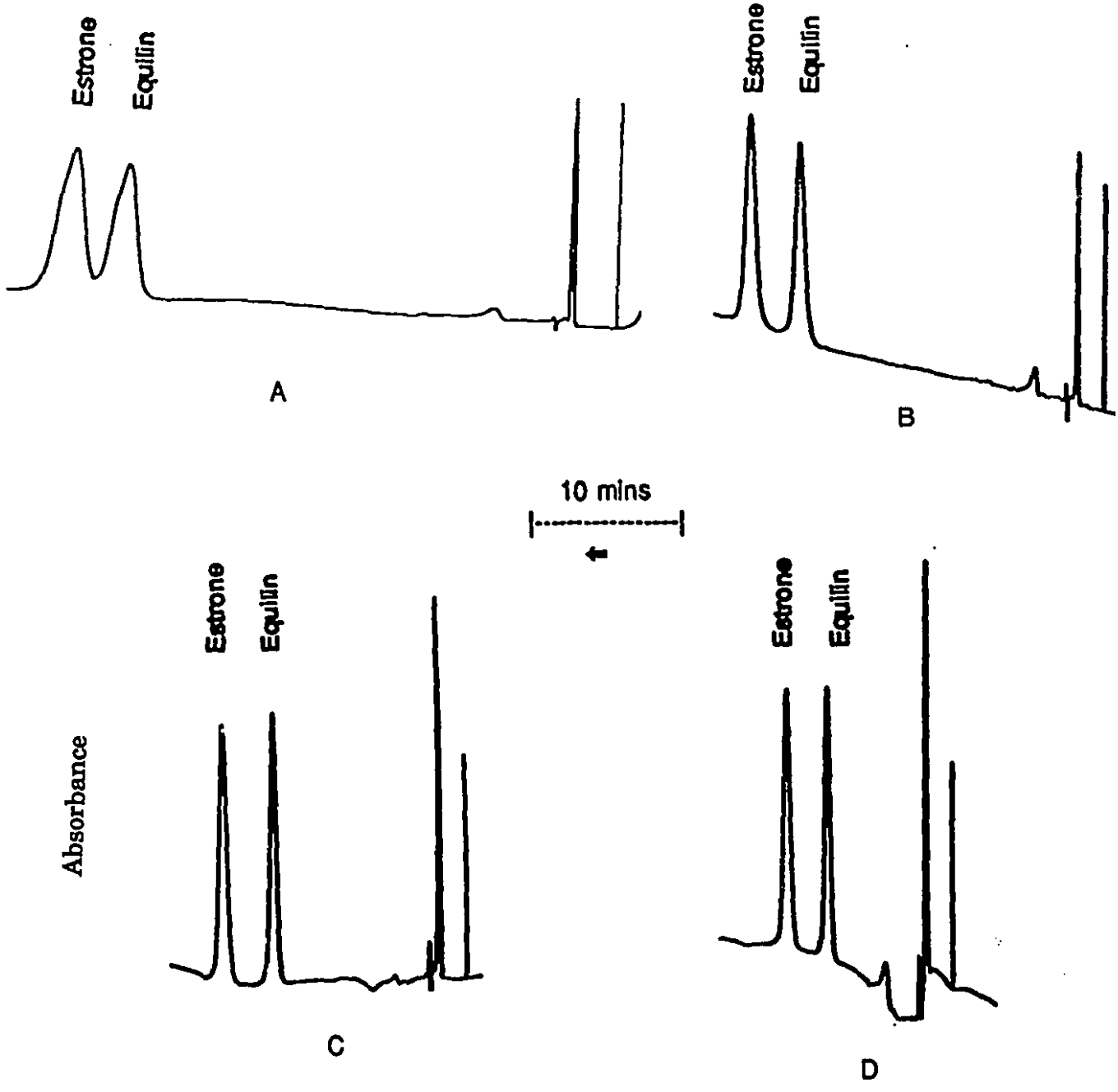

Figure 4.5 Effect of cyclodextrin concentration on the separation of equilin and estrone. (A) 0 mg/ml; (B) 1 mg/ml; (C) 5 mg/ml; (D) 13 mg/ml methyl-β-cyclodextrin. Mobile phase is 45:55 0.05M KH_2PO_4.

Table 4.4 summarizes the effect of DM-β-cyclodextrin on the separation and resolution factors for equilin and estrone. As can be seen, there is a significant increase in separation and resolution factors until the DM-β-cyclodextrin concentration reaches 10 mg/ml. At this point, the further addition of cyclodextrin had a minimal affect on resolution despite a continued reduction in capacity factor. Each steroid has a different affinity for the cyclodextrin cavity, thus varying the concentration will alter the elution rate of each solute through the column.

TABLE 4.4

Effect of DM-β-Cyclodextrin Concentration on Separation of Equilin and Estrone

[DM-β-CD] (mg/ml)	α	Rs
0.0	1.11	1.25
1.0	1.16	2.34
2.0	1.21	2.70
5.0	1.31	2.94
10.0	1.38	3.05
13.0	1.38	3.06

* All mobile phases were composed of 45:55 0.05M KH_2PO_4:methanol with the indicated amount of cyclodextrin.

The difference in inclusion complex strength can be attributed to the presence of the double bond on the B-ring in equilin. This double bond, absent in estrone, gives equilin a more rigid structure compared to estrone. This suggests that the steroid geometry plays a role in inclusion complex formation as the double bond is the only difference between the two compounds. The more rigid structure of equilin forms a stronger inclusion complex than the more flexible estrone.

The effect of varying the concentration of all three β-cyclodextrins on the separation of estrone and its hydroxy derivatives was also studied. Figure 4.6 graphs the effect of concentration of the three different types of cyclodextrins on the resolution of 2- and 4-hydroxyestrone.

Native β-cyclodextrin had the greatest effect on resolution, achieving a dramatic increase in resolution. However, due to its limited solubility, the maximum β-cyclodextrin mobile phase concentration was restricted at this methanol concentration to 6 mg/ml. Increases in separation were also noted for DM-β-cyclodextrin and HE-β-cyclodextrin as mobile phase additives, but not to the same extent as the native β-cyclodextrin. This phenomenon is in contrast to the separation of estrone and equilin, where derivatization of the cyclodextrin resulted in an improvement in separation. This indicates that hydrogen bonding interactions between the rim hydroxyl groups and the hydroxyl groups from 2- and 4-hydroxyestrone plays a critical role in selectivity. When the hydroxyl groups are replaced with hydroxyethyl groups, selectivity is reduced as the hydroxyl groups on the cyclodextrin are now further away from the rim. Relative selectivity decreases even further when β-cyclodextrin is derivatized with methyl groups. The replacement of the hydroxyl groups significantly reduces the hydrogen bonding interactions.

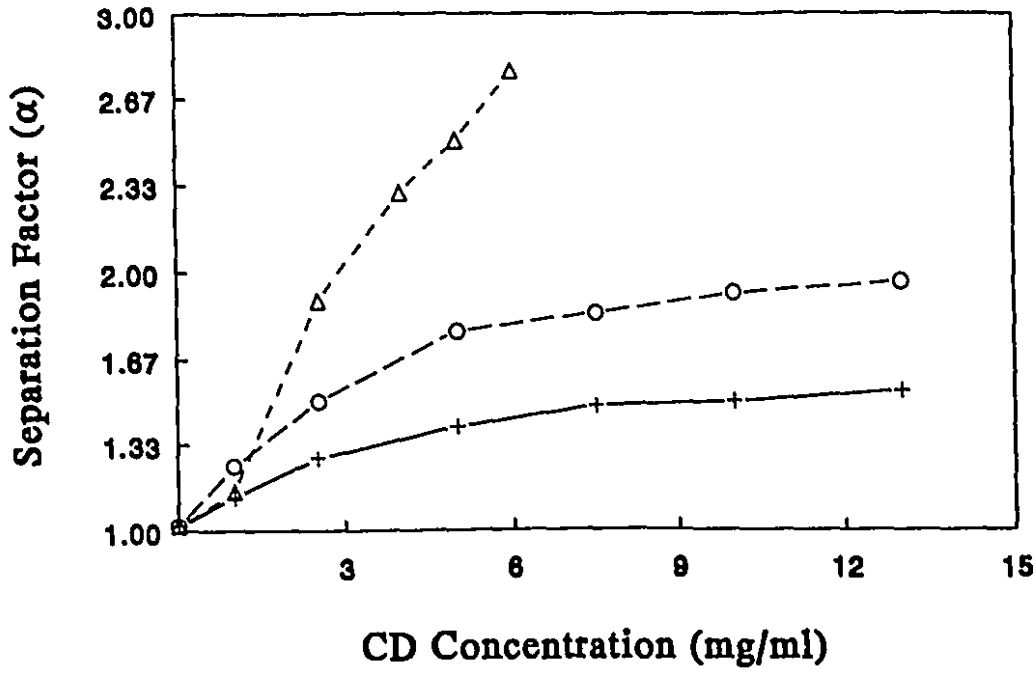

Figure 4.6 Effect of cyclodextrin concentration of the separation of 2- and 4-hydroxyestrone. Mobile phase is 60:40 0.05M KH_2PO_4:methanol plus indicated amount of cyclodextrin. + = DM-β-CD; Δ = β-CD; O = HE-β-CD.

Using the relationship between capacity factor and cyclodextrin concentration developed by Fujimura *et al.* [15], we can calculate the apparent formation constant (K_f) for the inclusion complex from the following equation:

$$\frac{1}{k'} = \frac{1}{k_0'} + \frac{[CD]\,K_f}{k_0'} \qquad (4.1)$$

where [CD] is the cyclodextrin concentration in the mobile phase and k_0' is the capacity factor for a solute that does not form an inclusion complex. A plot of 1/k' *versus* cyclodextrin concentration gives a straight line from whose slope the formation constant can be determined. It should be noted that these apparent formation constants are dependant on the methanol concentration in the mobile phase, as a change in the mobile phase polarity will affect the inclusion complex strength [8]. Table 4.5 summarizes the apparent formation constants of estrone and its derivatives at 45% v/v methanol with the three types of cyclodextrin studied.

TABLE 4.5

Apparent Formation Constants for Estrone and it's Derivatives

	K_f (M)			
Mobile Phase Additive	Estrone	2-HE	4-HE	16α-HE
β-Cyclodextrin	622	409	1138	482
DM-β-Cyclodextrin	713	481	849	466
HE-β-Cyclodextrin	376	318	714	278

* All mobile phases were composed of 55:45 0.05M KH_2PO_4:methanol

Three of the four steroids formed a stronger inclusion complex with the unmodified β-cyclodextrin while 2-HE formed a stronger complex with the DM-β-CD. In all cases, inclusion complex strength was weaker with the HE-β-cyclodextrin than with the other two forms. The difference in inclusion complex strength between 2- and 4-HE suggests that inclusion complex formation occurs with the A-B rings of the steroids. However, the differences in inclusion complex strength between estrone and 16α-hydroxyestrone implies that an inclusion complex also forms with the C-D rings, so selectivity can be based on differences in both ring systems. Note, however, that inclusion complex strength alone cannot be used to predict retention in this system as the solutes may also interacting distinctively with the stationary phase.

4.3.4 Effect of Methanol Concentration

The influence of methanol concentration on the separation and retention of equilin and estrone as well as estrone and its derivatives was studied while keeping the cyclodextrin concentration constant. The retention profiles of all five steroids followed the reverse-phase model where the capacity factor of all the steroids decreased with increasing mobile phase methanol concentration [16]. The resolution of equilin and estrone also decreased with increasing methanol concentration. Increasing the organic content of the solvent will weaken the strength of the inclusion complex formed between the guest and the cyclodextrin [17]. Another point to consider is that the methanol in the mobile phase may compete with the solute for inclusion complexation. Alcohols have been found to form inclusion complexes with β-cyclodextrin [18]. This competition is usually minor however it becomes more significant at higher methanol concentrations. Inclusion complexation can be given by:

$$S+CD \rightleftharpoons S-CD \qquad (4.2)$$

where S is the substrate, CD is cyclodextrin, and S-CD is the inclusion complex. The formation constant for the inclusion complex can be expressed as:

$$K_f = \frac{[S\text{-}CD]}{[S]\ [CD]} \qquad (4.3)$$

Similarly, the formation constant for methanol can be defined as:

$$K_{MeOH\text{-}CD} = \frac{[MeOH\text{-}CD]}{[MeOH]\ [CD]} \qquad (4.4)$$

Assuming a 1:1 stoichiometry between the cyclodextrin and methanol, the true formation constant for the solute, K', can be given by:

$$K' = \frac{K_f}{1 + K_{MeOH}[MeOH]} \qquad (4.5)$$

As can be seen from equation 4.5, higher methanol concentrations will compete more for the cyclodextrin cavity and hence decrease the K_f and the strength of the inclusion complex.

Figure 4.7 shows the effect of methanol concentration on the separation of 2- & 4-HE and 4- & 16α-HE using DM-β-cyclodextrin as the mobile phase additive. The separation of the 2- and 4-hydroxyestrone isomers declines with increasing methanol concentration until there is no separation at 60% methanol content. For the separation of 4- and 16α-HE, however, the reduction in retention is not accompanied by a loss in separation as the methanol concentration did not alter their relative inclusion complex strengths.

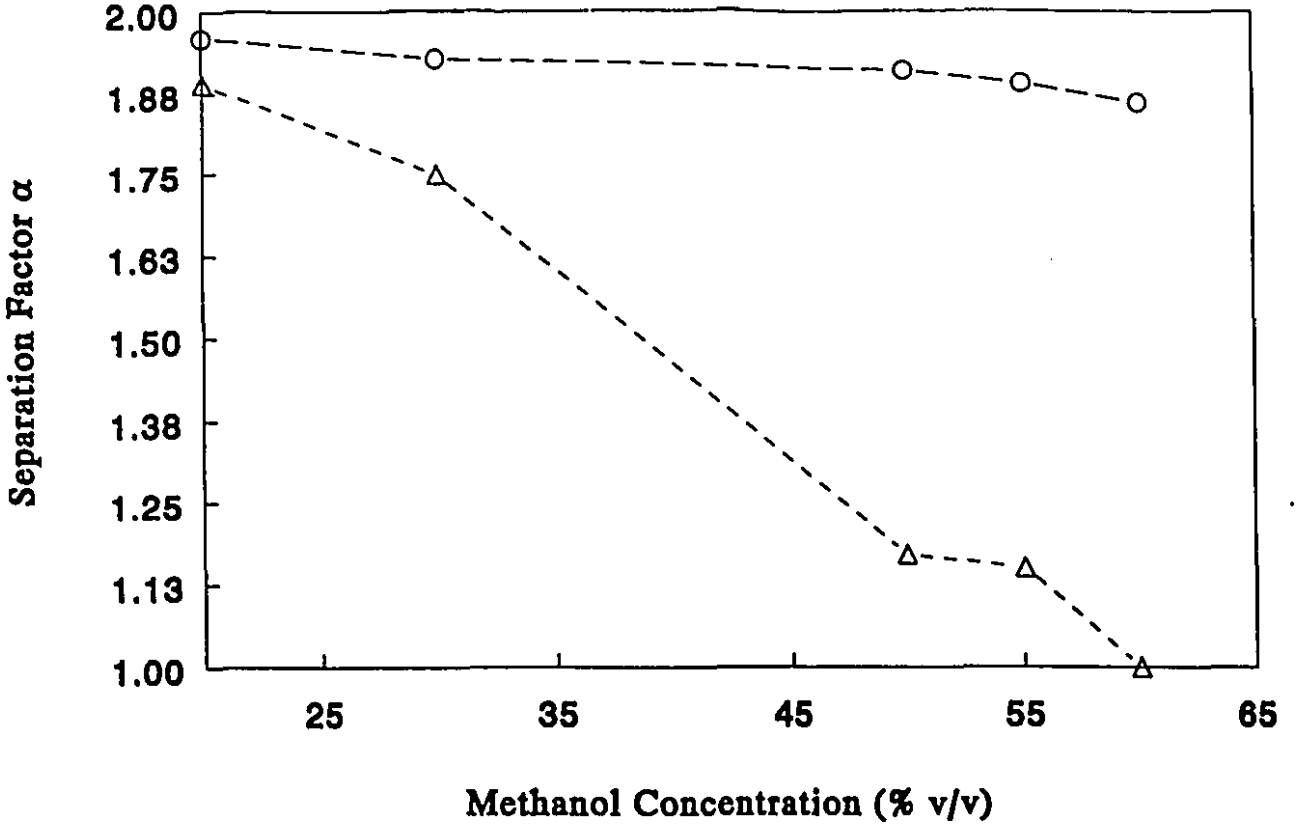

Figure 4.7 Effect of methanol concentration on the separation of 2- & 4-hydroxyestrone and 4- & 16α-hydroxyestrone. Mobile phase contains 4 mg/ml DM-β-cyclodextrin. O = $\alpha_{4\text{-},16\alpha\text{-HE}}$; Δ = $\alpha_{2\text{-},4\text{-HE}}$.

4.3.5 Effect of pH

The effect of varying the pH of the mobile phase from 3.2 to 8.0 on the retention and separation of the steroids was investigated. This pH was chosen as it is the range at which the silica-bonded C_{18} column is stable. Table 4.6 lists the capacity factors for estrone and its derivatives at different pH values. The mobile phase was kept constant with a composition of 60:40 0.05M KH_2PO_4:methanol and 5 mg/ml of DM-β-cyclodextrin.

The pH over this range has no effect on retention times as the capacity factors are nearly constant over the pH range studied here. The same trend was observed for the system used for the equilin/estrone analysis. This can be explained by the fact that the phenolic OH group of the estrogens has a pK_a of 10 to 10.5 [4] so therefore they will stay predominantly in the protonated form at this pH range. As well, the phenolic OH groups on β-cyclodextrin has a pK_a of around 12.0 [19], a value above the pH range studied. Since all the species remain in the same form, the retention of these steroids will be unaffected by pH. Since the pH had no effect on retention, it consequently had no effect on separation. It is worthy to note, though, that the pH begins to have a slight effect at the upper pH value of 8.0 as partial ionization begins to increase the retention of the steroids. Increasing the pH above the upper limit of would have resulted in greater retention times as the solutes become ionized.

TABLE 4.6

Effect of pH on Capacity Factors

Steroid	Capacity Factor at Different pH					
	3.2	4.0	4.8	5.4	6.2	8.0
Estrone	20.8	20.9	20.7	21.1	21.4	22.5
2-Hydroxyestrone	12.4	12.6	12.5	13.2	13.3	13.9
4-Hydroxyestrone	9.44	9.54	9.70	9.90	10.0	10.7
16α-Hydroxyestrone	5.40	5.50	5.50	5.50	5.50	5.89

4.3.6 Effect of Buffer Concentration

The effect of mobile phase buffer concentration was studied by varying the KH_2PO_4 concentration from 0.0 to 0.1M while keeping the methanol and cyclodextrin concentrations constant. The mobile phase composition was 60:40 KH_2PO_4:methanol with 5 mg/ml DM-β-cyclodextrin. Table 4.7 lists the capacity factors for estrone and hydroxyestrone derivatives at various buffer concentrations. As was the case with pH, buffer concentration had little effect on retention, with the exception of retention in the absence of any buffer. Usually, a molecule's retention time can be affected by changing the buffer concentration of the mobile phase, as its activity coefficients will depend on ionic strength [20]. However, since the pH of this mobile phase was 5.5, the steroids are not ionized and therefore are not affected by ionic strength.

TABLE 4.7

Effect of KH_2PO_4 Concentration on Capacity Factors

Steroid	Capacity Factor at Different KH_2PO_4 Concentrations					
	0.00	0.01	0.025	0.05	0.075	0.10
Estrone	23.0	18.0	18.1	18.5	19.1	20.1
2-Hydroxyestrone	15.5	11.1	11.3	11.4	11.8	12.5
4-Hydroxyestrone	11.8	8.40	8.57	8.80	9.00	9.50
16α-Hydroxyestrone	7.40	4.90	4.94	5.06	5.20	5.46

The capacity factor with no KH_2PO_4 was greater than when buffer ions are present in the mobile phase. This can attributed to the fact that phosphate has been to known to interact favourable with solute molecules so as to minimize adsorption of solute molecules on unreacted silanol group in the stationary phase [21,22] thus reducing their retention times.

Since buffer concentration had no effect on capacity factor, it consequently had no effect on the separation of equilin and estrone or estrone from its derivatives.

4.4 CONCLUSIONS

Cyclodextrins as a mobile phase additive have proven to be useful in the separation of two similarly structured steroids, equilin and estrone. There was an increase in resolution when β-cyclodextrin was used as a mobile phase additive. There was a further increase in resolution when the cyclodextrin was modified with methyl or hydroxyethyl groups. β-Cyclodextrin, DM-β-cyclodextrin, and HE-β-cyclodextrin were also suitable as mobile phase additives in the separation of estrone, 16α-hydroxyestrone, 2-hydroxyestrone, and 4-hydroxyestrone. The native β-cyclodextrin proved to be superior than the two derivatized β-cyclodextrins for their separation. It was concluded that inclusion complexes can be formed with the A-B rings and/or with the C-D rings of the steroids.

Increasing the cyclodextrin concentration in the mobile phase resulted in greater selectivity and reduced capacity factors. It was also found that retention and selectivity decreased with increasing methanol content and was unaffected by pH and buffer concentration.

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CHAPTER 5

COMPARISON OF THE SEPARATION OF FAT-SOLUBLE VITAMINS USING β-CYCLODEXTRINS IN HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND MICELLAR ELECTROKINETIC CHROMATOGRAPHY

5.1 INTRODUCTION

Vitamins are molecules essential for normal metabolic processes. They are required for catalytic purposes and function as co-enzymes, prosthetic groups on proteins, and hormones. Vitamins can be divided into two categories:

1) *Fat-soluble*: Also called lipid-soluble vitamins, they are very hydrophobic and are not soluble in water. They are all similarly structured and are comprised, entirely or separately, of 5-carbon isoprenoid units derived initially from acetyl CoA in plant and animal species capable of their biosynthesis. They include retinol (A), ergocalciferol (D_2), cholecalciferol (D_3), α-tocopherol (E), 3-phytylmenadione (K_1), menatetrenone (K_2), and menadione (K_3).
2) *Water-soluble*: These vitamins are water-soluble and their structures have little in common. This group includes thiamine

(B_1), riboflavin (B_2), pyridoxine (B_6), cyanocobalamin (B_{12}), ascorbic acid (C), biotin (H), folic acid (M), niacin, and pantothenic acid.

Fat-soluble vitamin analysis by HPLC is usually performed in the normal phase due to the molecules' considerable hydrophobic character [1-3], although vitamins D_2 and D_3 can be separated effectively using reversed-phase methods [4]. Reversed phase HPLC for these molecules is not ideal as they typically have very high capacity factors and therefore suffer from excessive band broadening due to longitudinal diffusion. However, the preference of reversed-phase techniques over normal phase ones demonstrates the need to develop reversed-phase methods for analytes that are usually performed in the normal phase.

There is sufficient evidence that fat-soluble vitamins form inclusion complexes with β-cyclodextrin as a variety of vitamin-CD complexes have been synthesized and characterized [5-7]. This is especially true of vitamins D_2 and D_3, whose steroid-like structure make them suitable for inclusion complexation (see chapter 4). To date, only a minimal amount of work has been performed using cyclodextrins for vitamin analysis by HPLC. El-Gizawy and co-workers [8] separated various water-soluble vitamins using a β-cyclodextrin stationary phase while Abidi and Mounts [9] separated α-, β-, γ-, and δ-tocopherol on β- and γ-cyclodextrin columns using a non-aqueous mobile phase.

It will be shown that the addition of cyclodextrins to the mobile phase in reversed phase HPLC can improve the separation and analysis of fat-soluble vitamins. Inclusion complex formation will increase the vitamin's affinity for the mobile phase thus reducing retention times and band broadening thus improving their overall chromatography. The vitamins under investigation include vitamins A, D_2, D_3, E (all four isomers), E acetate, K_1, K_2, and K_3. The first part of this chapter will discuss their resolution by HPLC. The choice of

stationary phase will be discussed. As well, separations obtained with three types of derivatized β-cyclodextrins will be compared.

Recent advances in capillary electrophoresis has resulted in the development of a new analytical technique that is complementary to HPLC. The innovation of micellar electrokinetic chromatography (MEKC), introduced in chapter 2, has allowed for the analysis of uncharged molecules using a charged micellar pseudo-phase. The most common micelle used in MEKC is sodium dodecyl sulfate (SDS), an anionic micelle with significant hydrophobic character, making them ideal for the capillary electrophoretic analysis of fat-soluble vitamins.

A limited amount of research has been carried out using capillary electrophoresis in the analysis of vitamins, mostly on the water soluble variety. Jegle [10] separated water-soluble vitamins by CZE while Ma and co-workers [11] used the same CE mode to look at a retinol (vitamin A)-protein complex using laser fluorescence. Huopalahti and Sunell [12] separated the B series vitamins by CZE. MEKC has also been used in vitamin analysis. Nishi and co-workers [13] separated 11 water soluble vitamins using MEKC while Boonkerd and co-workers [14] used it to separate the vitamins of the B group. Yik and co-workers [15] used MEKC to analyze a mixture of B_6 vitamers while using amperometric detection. Ong and co-workers [16] separated nine vitamins using micellar electrokinetic chromatography with β- and γ-cyclodextrin in the buffer. The only two fat-soluble vitamins studied here were vitamins A and α-tocopherol.

The second part of this chapter will investigate the separation of various fat-soluble vitamins using MEKC and several β-cyclodextrins as buffer additives. The effect of cyclodextrin and buffer organic modifier on separation and migration will be discussed. Finally, the separations obtained by MEKC will be compared to those acquired by HPLC.

5.2 INSTRUMENTAL

5.2.1 Apparatus

The high performance liquid chromatographic system was described in section 3.2.1. Separations were performed on a Zorbax ODS (150 x 4.6 mm I.D.) or a phenyl column (100 x 4.6 mm I.D.). Capillary electrophoresis was performed on an in-house CE system comprising of a Bertan Model 230R power supply (Bertan Associates, Hicksville, NY, USA) and an Isco CV^4 (Isco, Lincoln, NE, USA) UV/VIS detector. The output of the power supply was connected to the buffer reservoir via platinum electrodes (Bioanalytical Systems, West Lafayette, IN, USA). Separations were performed on fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 375 μm O.D. The capillary and buffer reservoirs were encased inside a plexiglass box whose lid was equipped with interlocking switches. When the lid was raised, the power supply was turned off. Electropherograms were acquired with the Waters System Interface Module (Millipore Corp. Milford, Mass, USA) and then processed on the Waters Maxima 820 Chromatography Workstation. A diagram of the CE system used can be found in Figure 1.3.

5.2.2 Chemicals

Ergocalciferol (D_2), cholecalciferol (D_3), α-tocopherol (E), β-cyclodextrin (β-CD), hydroxyethyl-β-cyclodextrin (HE-β-CD), heptakis(2,6-di-O-methyl)-β-cyclodextrin (DM-β-CD), and sodium tetraborate (borax) were obtained from Aldrich (Milwaukee, WI, USA). 3-Phytylmenadione (K_1), menatetrenone (K_2), menadione (K_3), and δ-tocopherol were purchased from Sigma (St. Louis, MO, USA). β-Tocopherol and γ-tocopherol were purchased from Matreya (Pleasant Gap, PA, USA). Ultra pure sodium dodecyl sulfate (SDS) was obtained from ICN Biochemicals (Montreal, QC, Canada). Sodium hydroxide and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Methanol was

purchased from Fisher (Montreal, QC, Canada). Potassium phosphate, ethanol, and HPLC grade acetonitrile were obtained from A&C Chemicals (Montreal, QC, Canada). Water was doubly distilled and deionized.

5.2.3 CE Procedures

The capillaries had a total length of 77 cm and a separation length of 55 cm and were conditioned prior to use with 1N NaOH for 10 minutes. Capillaries were rinsed between runs with 0.1N NaOH, distilled water, and then the running buffer for two minutes each. The detection wavelength was 280 nm. The pH of the running buffer was adjusted by the addition of appropriate amounts of boric acid, hydrochloric acid or sodium hydroxide. All buffers were filtered through a 0.45 μm membrane and then degassed prior to use. Samples were dissolved in the running buffer, degassed, and injected hydrodynamically at a height of 15 cm and the injection time was 8-10 seconds.

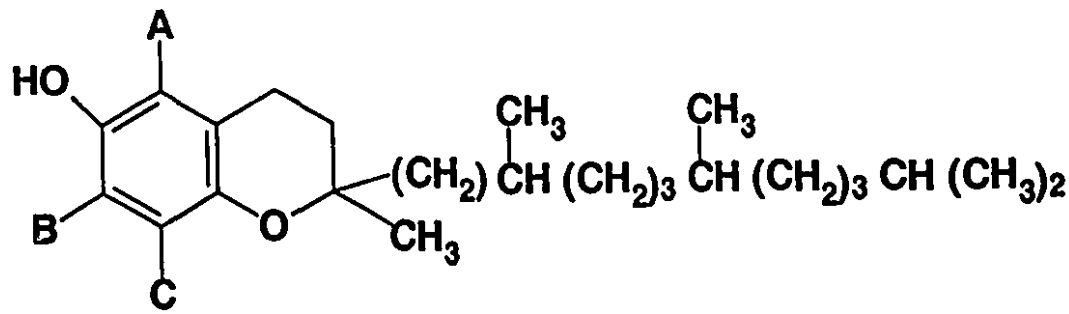
5.2.4 HPLC Procedures

The mobile phase was prepared by mixing methanol with water. Cyclodextrin was then dissolved and the mixture was degassed and filtered through a 0.45 μm membrane filter. The solutes were dissolved in methanol to give a concentration of about 1 mg/ml and the typical injection volume was 1 μl. The flow-rate was 1.0 ml/min. The wavelength of detection was 280 nm.

Retention times were determined by averaging at least three separate determinations. A reproducibility study was conducted where five injections had an RSD of less than 2.5% for the capacity factor and of less than 7% for the resolution factor.

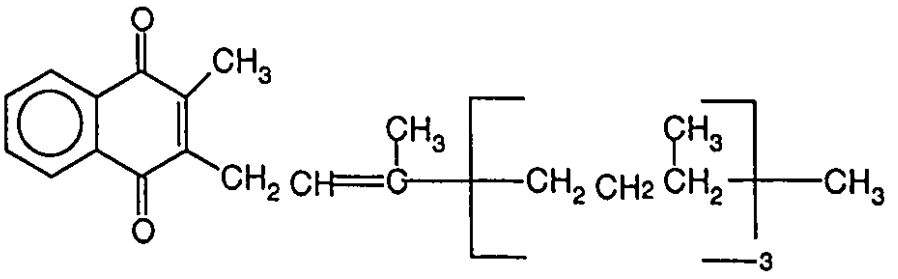
5.3 RESULTS AND DISCUSSION

Figure 5.1 shows the structures of the E vitamers, α-, β-, γ-, and δ-tocopherol. The structures for vitamins A, D_2, D_3, K_1, K_2, and K_3 are found in Figure 5.2.

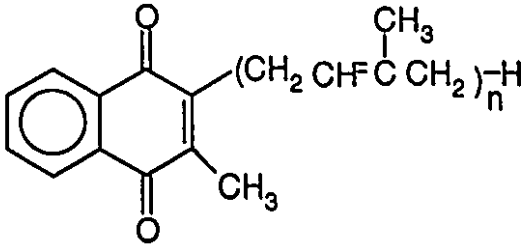


α-tocopherol (5,7,8-trimethyltocol): A=CH_3, B=CH_3, C=CH_3
β-tocopherol (5,8-dimethyltocol) : A=CH_3, B=H, C=CH_3
γ-tocopherol (7,8-trimethyltocol) : A=H, B=CH_3, C=CH_3
δ-tocopherol (8-dimethyltocol) : A=H, B=H, C=CH_3

Figure 5.1 Structure of vitamin E isomers.



1



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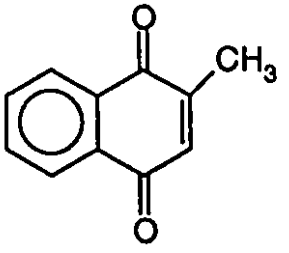

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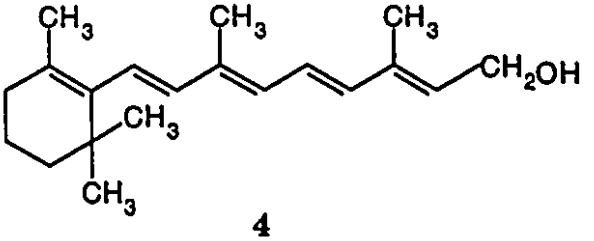

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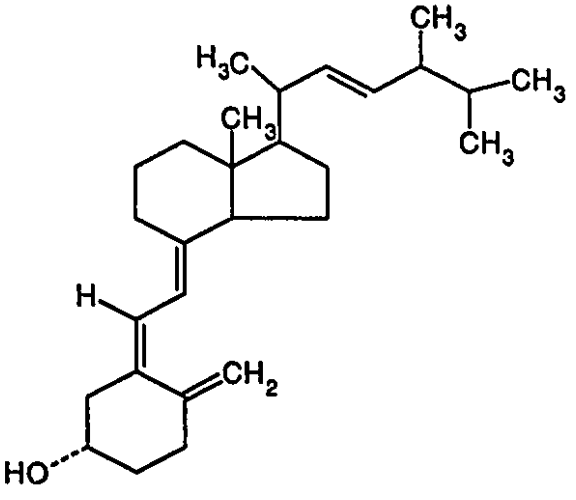

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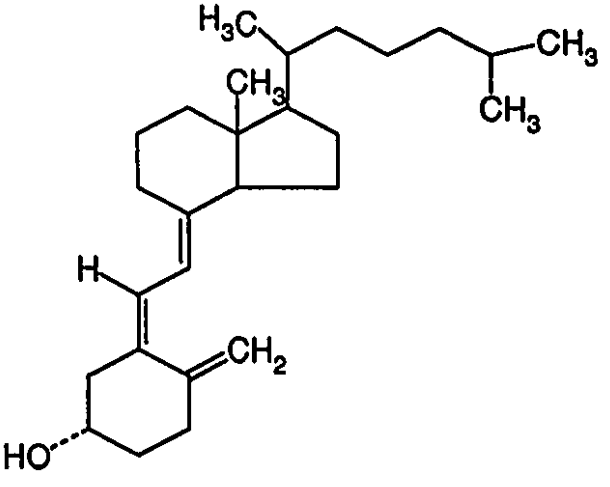


6

Figure 5.2 Structures of vitamins (1) K_1, (2) K_2, (3) K_3, (4) A, (5) D_2, (6) D_3.

5.3.1 Separation of Vitamins by HPLC

This section will discuss the separation of fat-soluble vitamins by reversed-phase HPLC. In most cases, the use of derivatized β-cyclodextrin improved their separation and reduced the retention times on reversed-phase stationary phases.

5.3.1.1 Preliminary Separations

Generally, fat-soluble vitamins are not analyzed using reversed-phase HPLC methods. An exception to this is the separation of vitamins D_2 and D_3 which can be separated on a C_{18} column using a very high concentration of methanol and acetonitrile in the mobile phase [4]. The main hurdle is that these molecules, because of their significant hydrophobic character, are retained very strongly by reversed-phase HPLC columns. The aim of the work presented in this section was to look at using β-cyclodextrin and derivatized β-cyclodextrins as mobile phase additives to increase the vitamins' affinity for the mobile phase as well aid in their separation. Inclusion complexation would reduce the amount of time spent in the stationary phase thus reducing retention times and band broadening.

Initial separations investigated the separation of vitamins A,D_2, D_3, and E (α-tocopherol). Figure 5.3 shows the separation of these four compounds on a C_{18} column. The mobile phase is composed of 90:10 methanol:water with no cyclodextrin. All four peaks are certainly separated; however their retention times had to be reduced in order to decrease the excessive band broadening of the latter 3 vitamins.

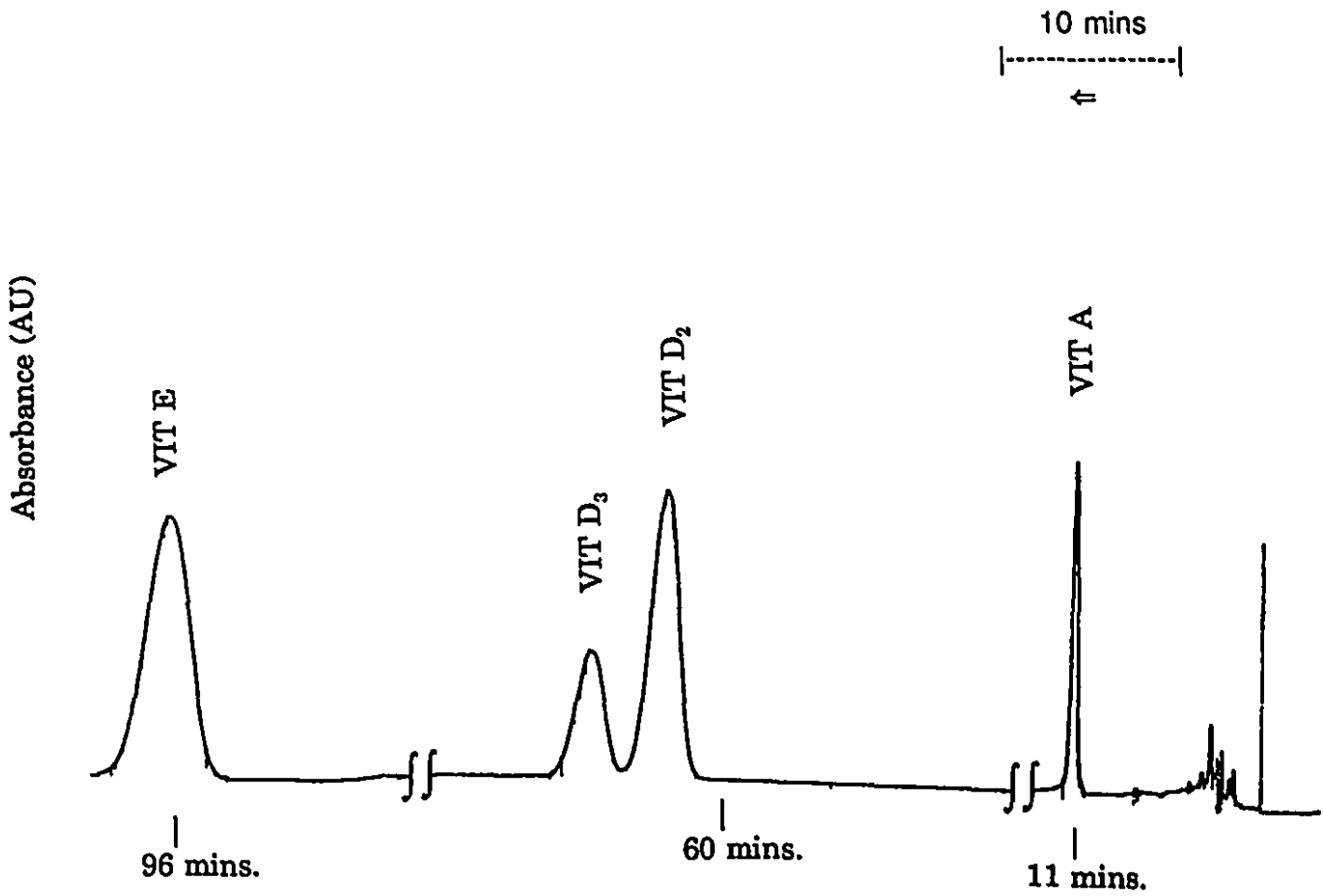


Figure 5.3 Separation of vitamins A, D_2, D_3, and E with no cyclodextrin in the mobile phase. Mobile phase was 90:10 methanol:water.

Initially, it was suspected that the high organic content of the mobile phase would deter inclusion complexation thus making cyclodextrins unsuitable for use as a mobile phase additive. However, this was found not to be the case as the addition of cyclodextrin to the mobile phase reduced the retention times of most of these compounds. Upon addition of 10 mg/ml of DM-β-cyclodextrin to the mobile phase, the retention times for vitamins D_2, D_3, E were significantly reduced and separation of D_2 and D_3 improved. This effect was not as prominent with vitamin A. This separation can be seen in Figure 5.4.

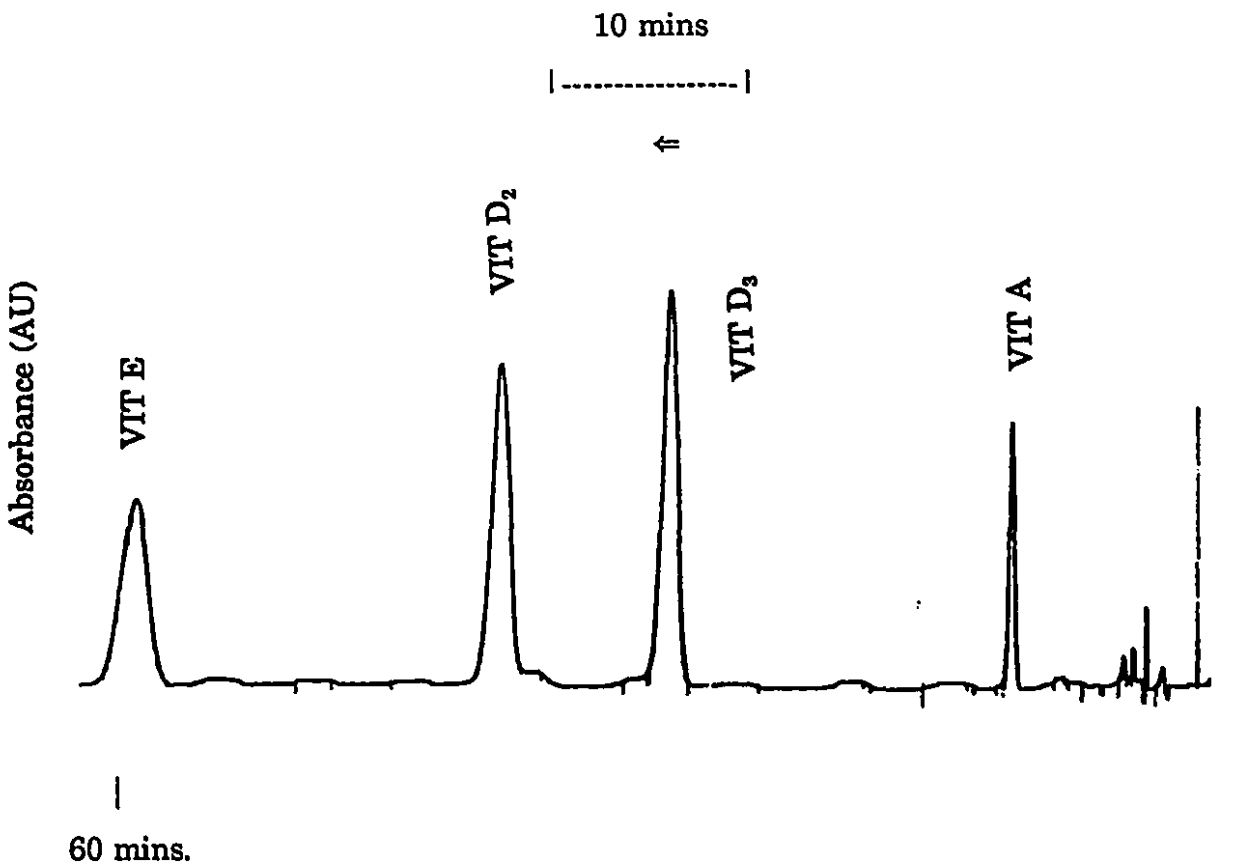


Figure 5.4 Separation of vitamins A, D_2, D_3, and E with 10 mg/ml DM-β-CD in the mobile phase. Conditions: 90:10 methanol:water.

Figure 5.5 shows the effect of varying the cyclodextrin concentration on the capacity factor of these four compounds.

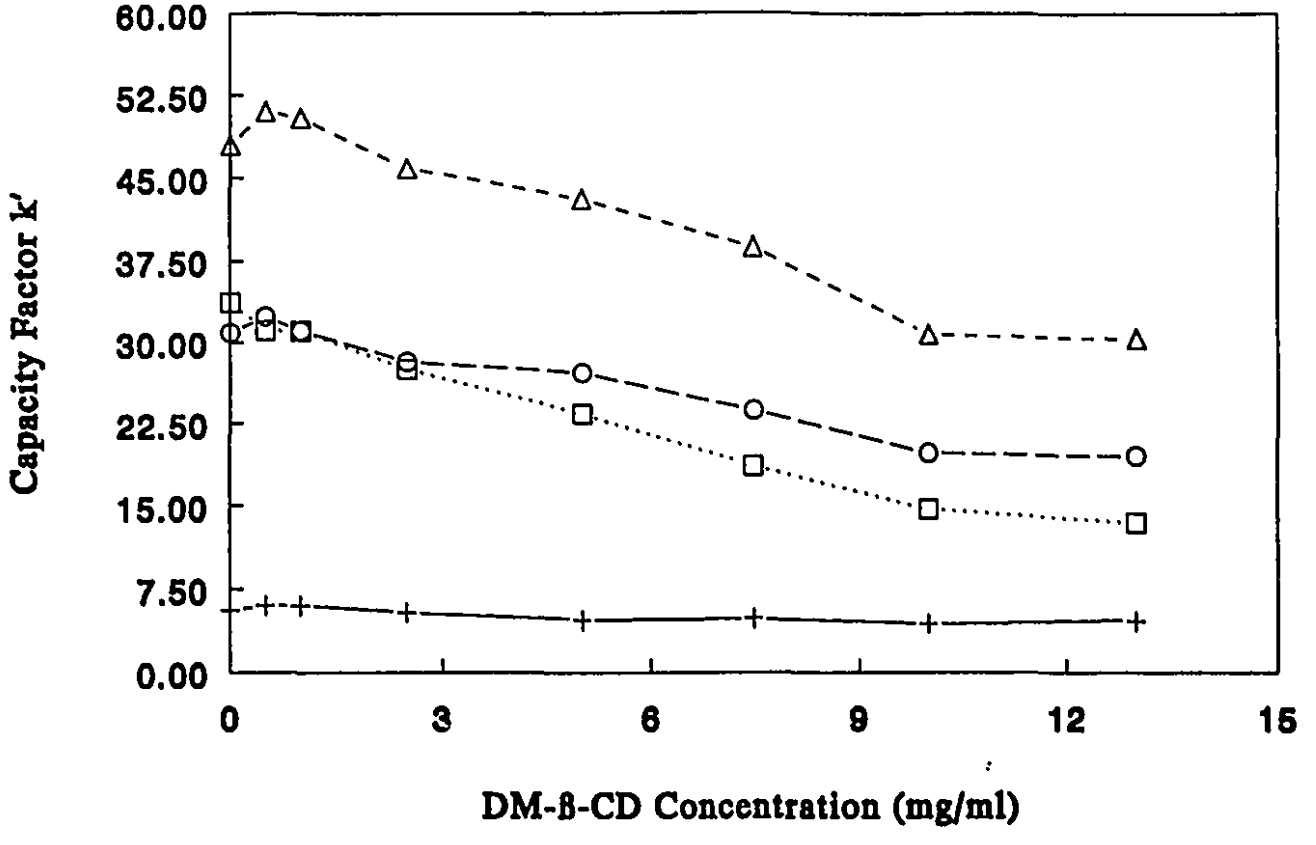


Figure 5.5 Effect of DM-β-CD concentration on capacity factor. Conditions: 92:8 methanol:water plus the indicated amount of DM-β-CD. Δ = vitamin E; o = D_2; □ = D_3; + = A.

As the cyclodextrin concentration increased, the capacity factor of vitamins D_2, D_3, and E decreased, indicating the formation of inclusion complexes. The vitamins now have a greater affinity for the mobile phase and therefore have a shorter elution time. This decrease in capacity factor was not observed for vitamin A. Increasing the water content to 15% did not have a significant effect on inclusion complexation with vitamin A. The capacity factor (k') of vitamin A with a mobile phase of 85:15 methanol:water was 30.1. The addition of 6 mg/ml of dimethyl-β-cyclodextrin to the mobile phase decreased the capacity factor to only 28.5. It was concluded that vitamin A does not form a strong inclusion complex with cyclodextrin in the mobile phase at these high organic concentrations. This is not really surprising as the size of the cyclohexene ring is probably too small to form a tight inclusion complex; therefore the relatively low polarity of the mobile phase would hinder strong inclusion complex formation. Regardless, vitamin A has a small enough capacity factor that it does not require the aid of cyclodextrin for its analysis. Vitamin A was not studied any further.

5.3.1.2 Separation of Vitamins D_2 and D_3

The addition of cyclodextrin to the mobile phase greatly improved the separation of vitamins D_2 and D_3 on a C_{18} stationary phase. Figure 5.6 shows the effect of dimethyl-β-cyclodextrin concentration on the separation of vitamins D_2 and D_3.

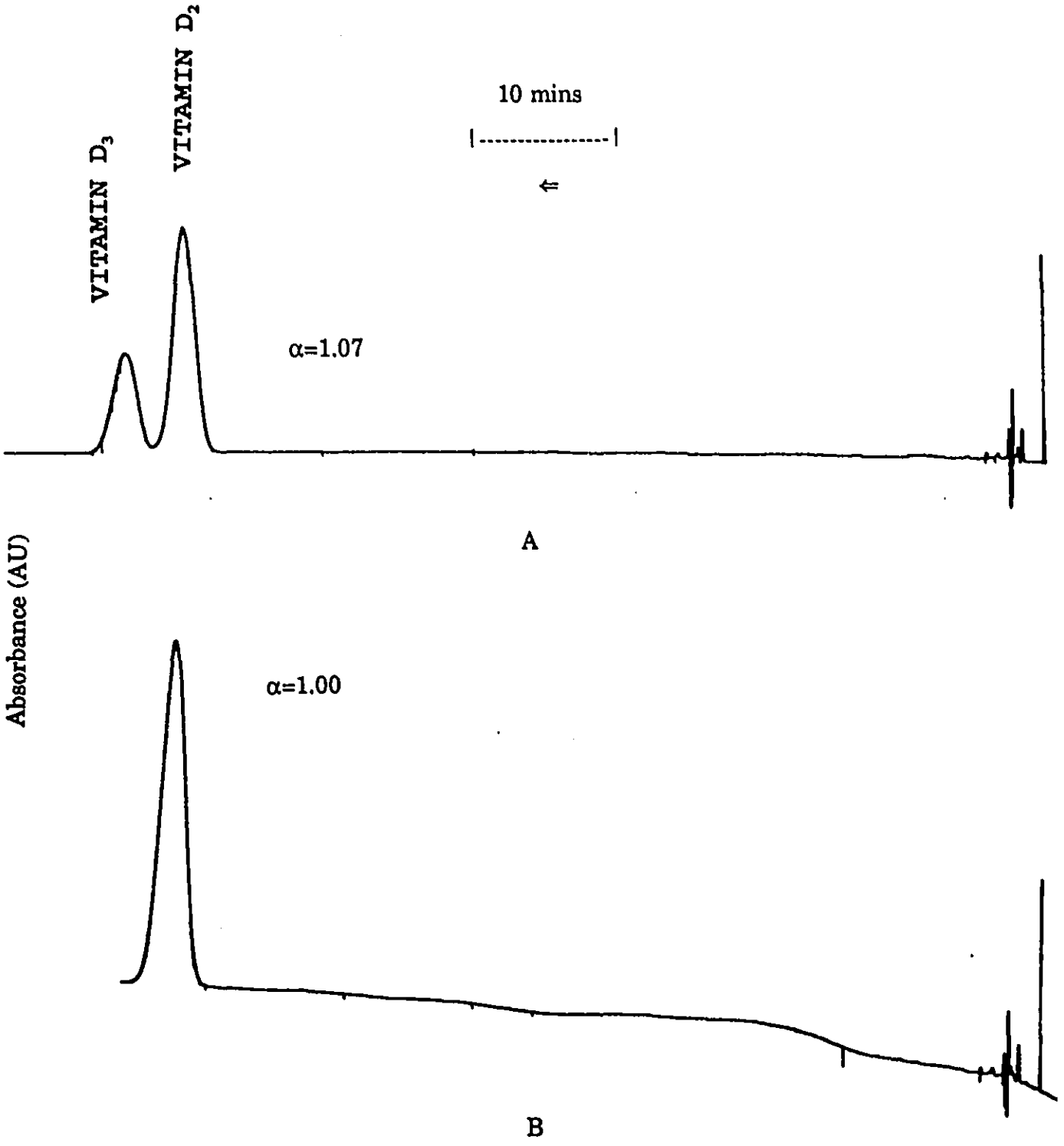


Figure 5.6 Effect of DM-β-CD concentration on the separation of vitamins D_2 and D_3. Mobile phase was 92:8 methanol:water plus (A) 0 mg/ml DM-β-CD; (B) 1 mg/ml DM-β-CD.

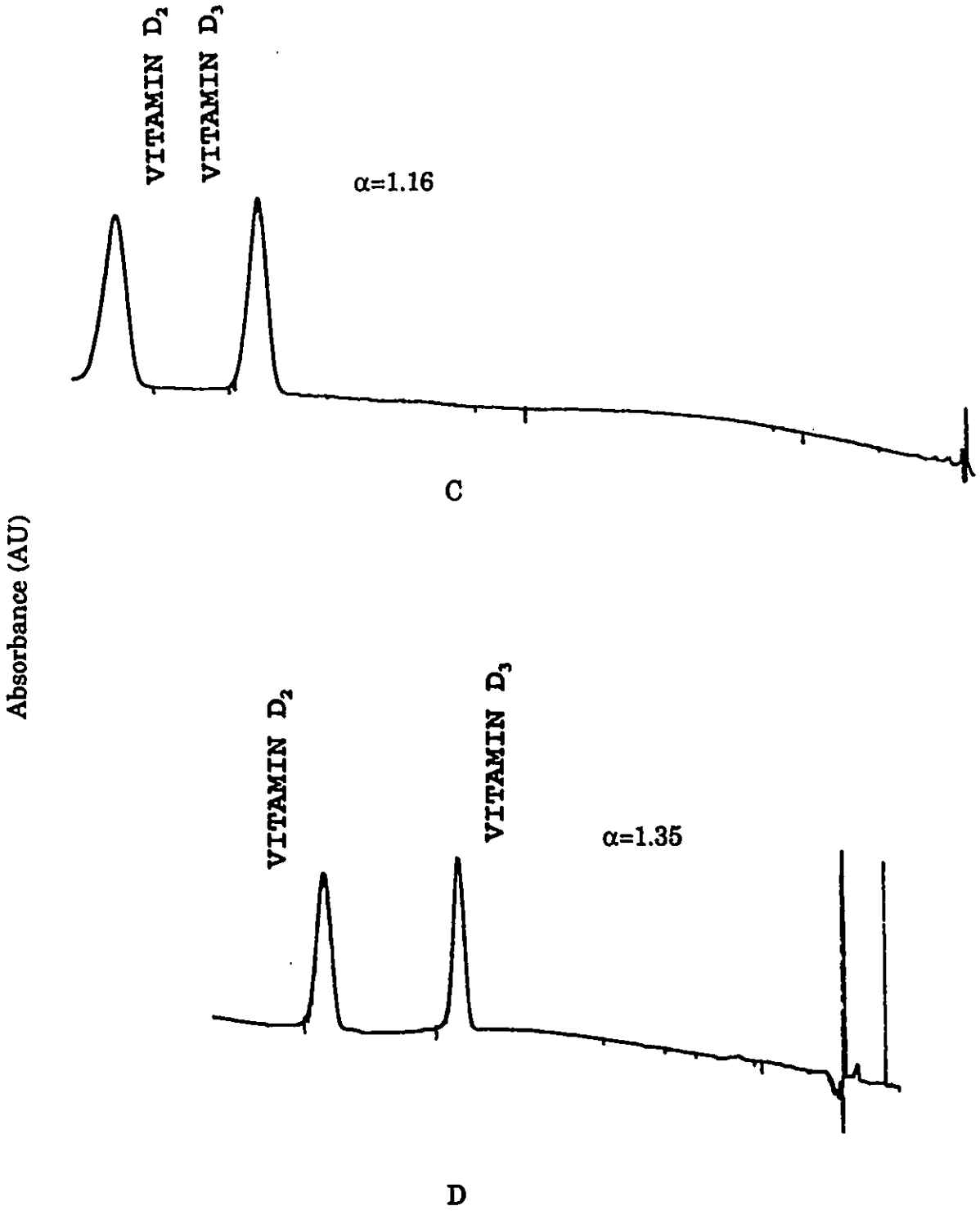


Figure 5.6 continued. Effect of DM-β-CD concentration on the separation of vitamins D_2 and D_3. Mobile phase was 92:8 methanol:water plus (C) 5 mg/ml DM-β-CD; (D) 10 mg/ml DM-β-CD.

Note that there is complete loss of resolution at a cyclodextrin concentration of 1 mg/ml. This was because vitamin D_3 formed a stronger inclusion complex with cyclodextrin than D_2; therefore, D_3 co-eluted with D_2 at this cyclodextrin concentration while the elution order was reversed at higher cyclodextrin concentrations.

Other derivatized β-cyclodextrins were also investigated in the separation of vitamins D_2 and D_3. Underivatized β-cyclodextrin was not soluble at these low mobile phase water concentrations (see Table 3.1). Figure 5.7 compares the separation of vitamins D_2 and D_3 with dimethyl-β-cyclodextrin, triacetyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin in the mobile phase. The DM-β-CD separated the two vitamins better than the other two, probably because the methyl groups on the cyclodextrin rim produce a more hydrophobic cavity than the hydroxyethyl or acetyl groups thus allowing for the formation of a stronger inclusion complex. Stronger inclusion complexation resulted in more time for interactions with the rim groups therefore resolution was better with the dimethyl-β-cyclodextrin.

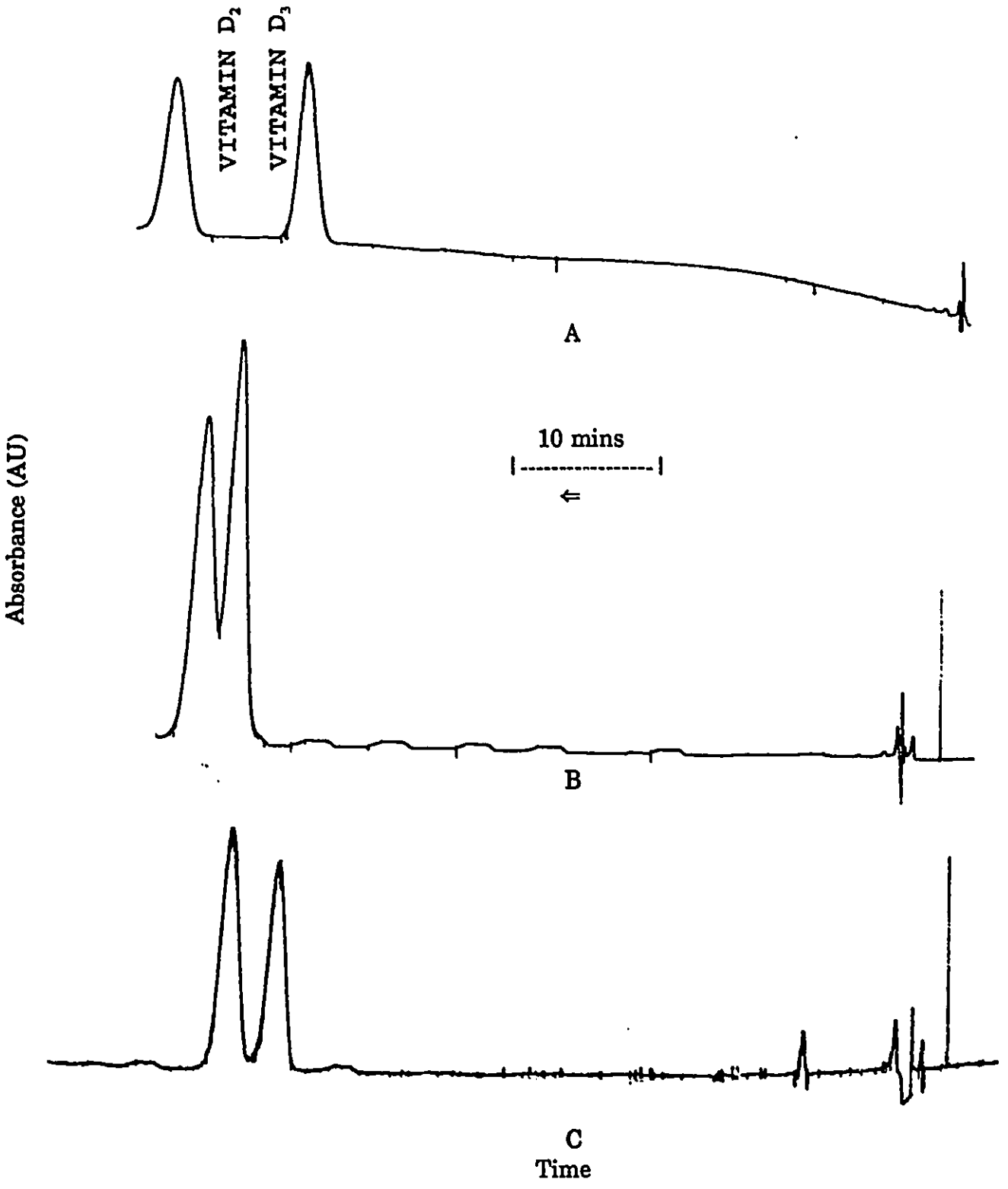


Figure 5.7 Comparison of different β-cyclodextrins in the separation of vitamins D_2 and D_3. Mobile phase was 92:8 methanol:water plus 5 mg/ml of (A) DM-β-CD; (B) HE-β-CD; (C) TA-β-CD.

5.3.1.3 Separations of Vitamin E

As was observed in section 5.3.1.1, vitamin E was strongly retained on a C_{18} stationary phase, even with cyclodextrin present in the mobile phase. Severe band broadening would make the separation of vitamin E isomers practically impossible with this stationary phase. It was decided to use a less hydrophobic stationary phase, namely a column having phenyl functional groups. A phenyl stationary phase is less hydrophobic than C_{18} and has slightly different selectivities due to the electron density of the aromatic ring [17]. Hydrophobic solutes will be retained to a lesser degree on a phenyl than a C_{18} column. This will reduce band broadening enough to make cyclodextrins useful as a mobile phase additive in the separation of vitamin E isomers.

This system was first used in the separation of vitamin E from vitamin E acetate, its water soluble derivative. Figure 5.8 shows the separation of the two vitamins with no cyclodextrin in the mobile phase.

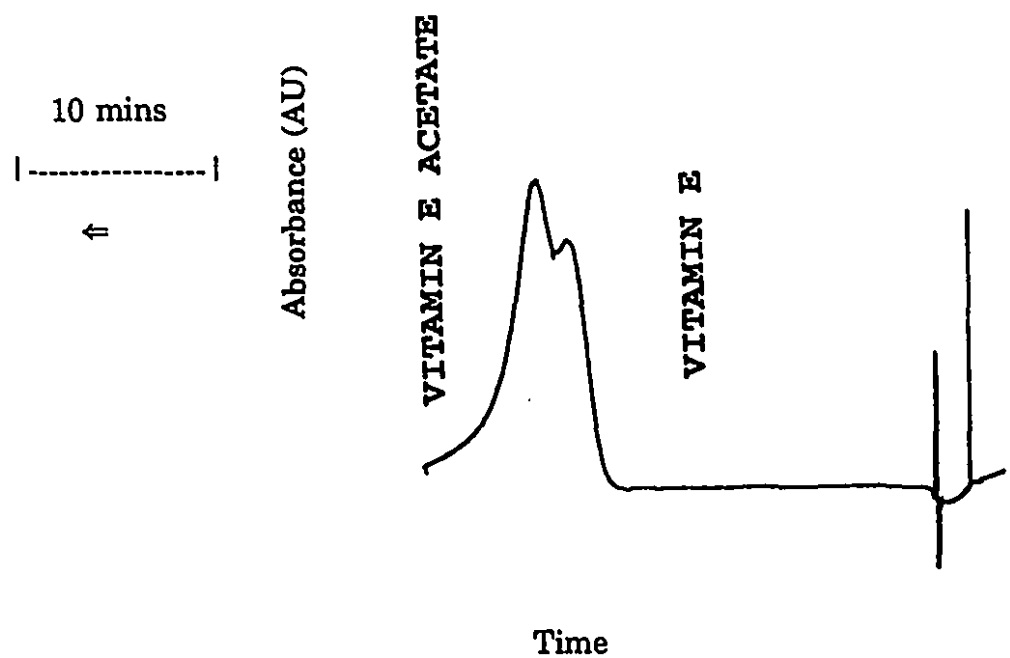


Figure 5.8 Separation of Vitamins E and E acetate on a phenyl column with no cyclodextrin. Mobile phase: 55:45 0.05M KH_2PO_4:methanol.

The resolution of the peaks here is poor; however, it is greatly improves upon the addition of cyclodextrin to the mobile phase. Figure 5.9 displays the improvement in separation with both β-cyclodextrin and dimethyl-β-cyclodextrin (DM-β-CD) in the mobile phase.

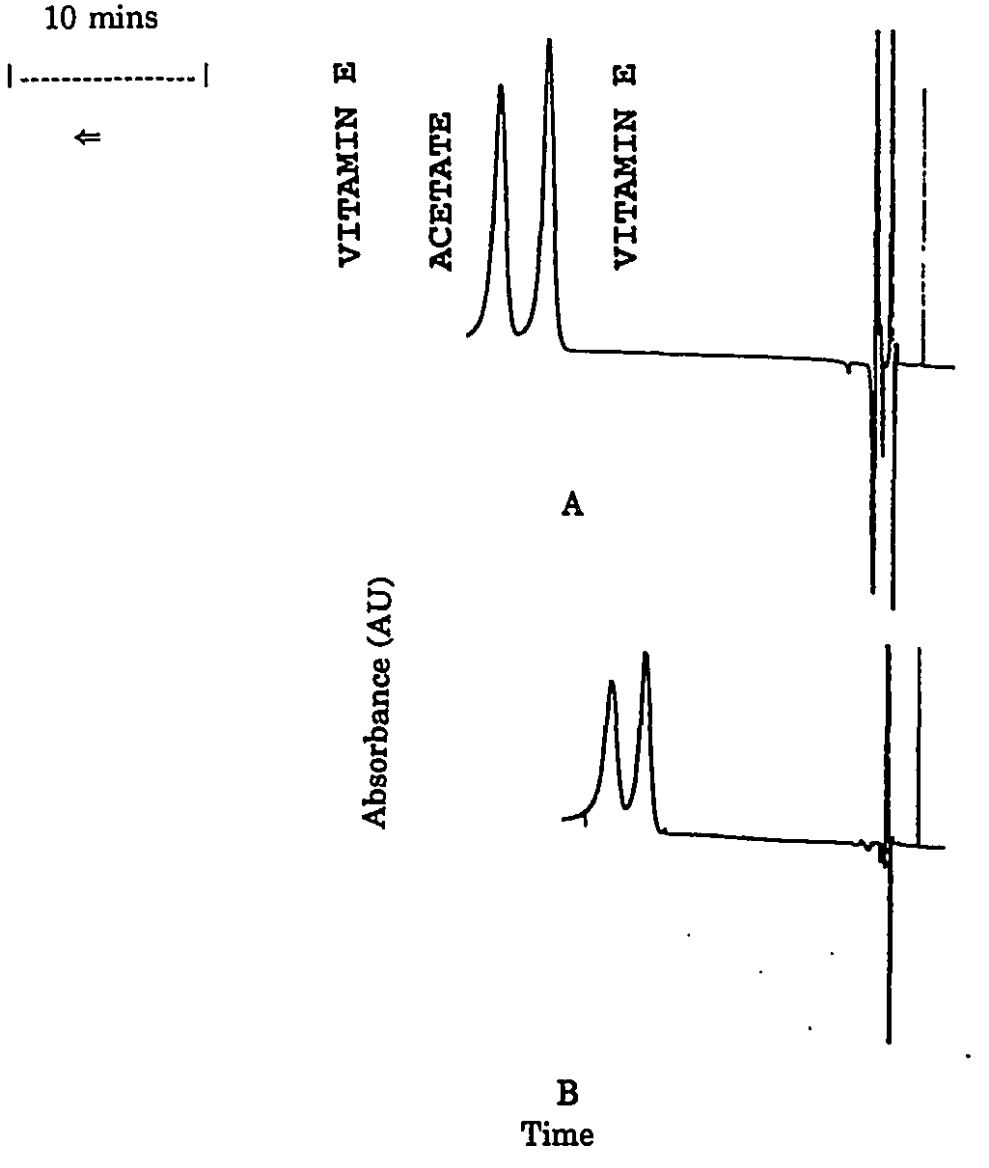


Figure 5.9 Separation of Vitamins E and E acetate on a phenyl column with (A) β-CD; (B) DM-β-CD in the mobile phase. Mobile phase: 55:45 0.05M KH_2PO_4:methanol plus 5 mg/ml of the indicated cyclodextrin.

The underivatized β-cyclodextrin was a useful additive as the aqueous content of the mobile phase was high enough for it to be dissolved. Both cyclodextrins gave similar separations for the two vitamins. At a concentration of 5 mg/ml, both cyclodextrins gave a separation factor of 1.14.

This reversed-phase system was then used to attempt to separate the four E vitamers, namely α-, β-, γ-, and δ-tocopherol, with β-cyclodextrin and dimethyl-β-cyclodextrin as mobile phase additives. This was a very challenging separation due to the difficulty in separation β- and γ-tocopherol. This difficult separation was first performed by normal-phase HPLC [18] while little progress has been reported with reversed-phase methods. As was mentioned in the introduction, Abidi and Mounts [9] separated them on β- and γ-cyclodextrin columns using a normal-phase mobile phase.

Figure 5.10 shows the attempt at separation all four E vitamers on a phenyl stationary phase. Despite much effort, the cyclodextrins were not able to distinguish between β- and γ-tocopherol. Both types of cyclodextrins certainly improved on the separation of the α- and δ-isomers; however they failed to resolve all four isomers. Differentiation of the vitamers by cyclodextrin would be on the basis of inclusion complex strength alone as there are no differences in the segment of the molecules that protrudes from the torus; therefore there will not be any variation in interactions with the rim groups. It is was found that the more hydrophobic isomers would form stronger inclusion complexes and therefore had shorter retention times. The successful separation of the four vitamers by micellar electrokinetic chromatography will be discussed in section 5.3.2.3.

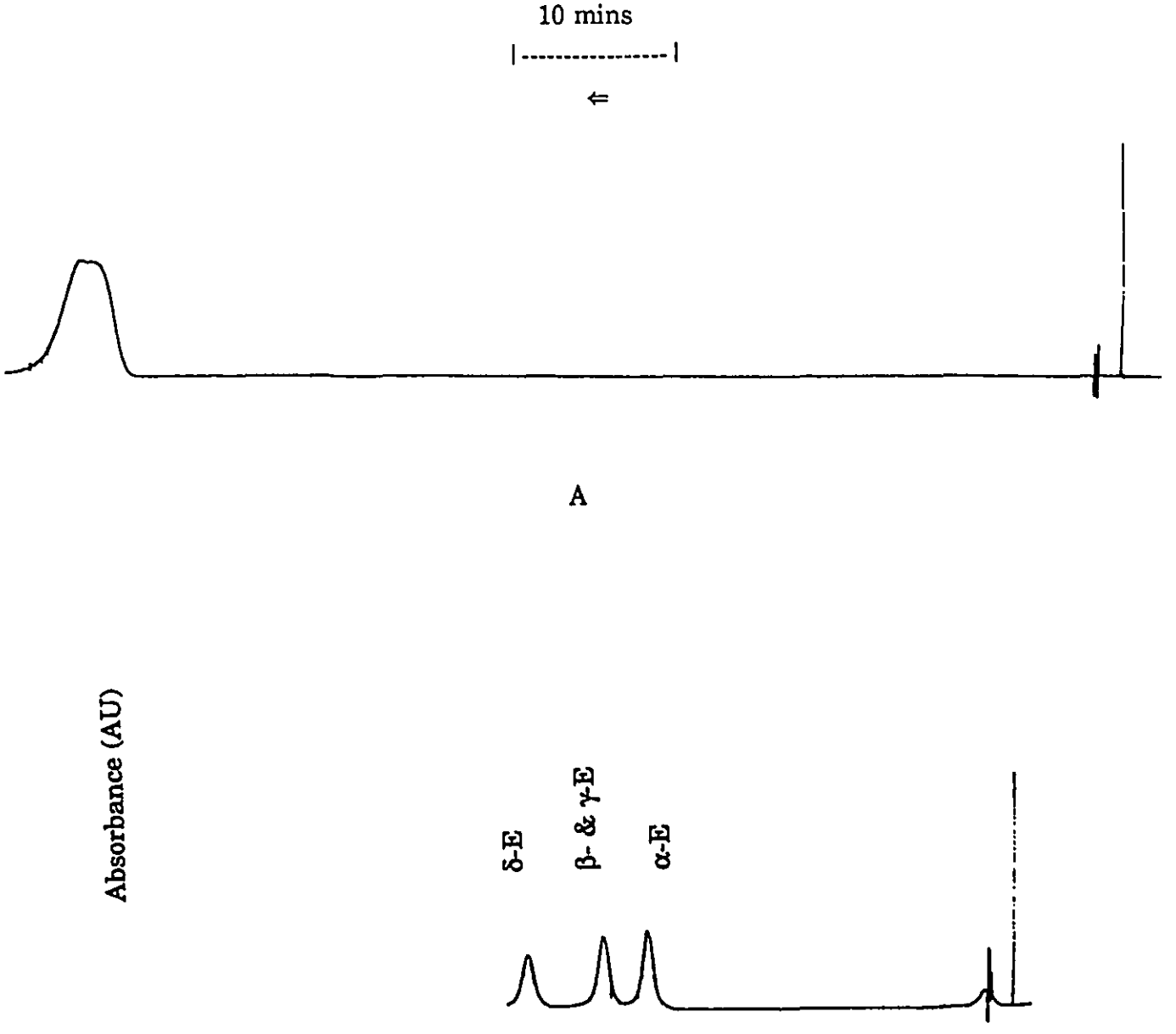


Figure 5.10 Separation of α-, β-, γ-, and δ-tocopherol. Conditions 65:35 0.05M KH_2PO_4, plus (A) 0 mg/ml; (B) 7.5 mg/ml DM-β-CD.

5.3.1.4 Retention Behaviour of the K Vitamins

The vitamins in the K series were studied to determine what effect β-cyclodextrin would have on their retention. Vitamins K_1 and K_2 are very hydrophobic molecules (see Figure 5.2) and are expected to be difficult to analyze using reversed-phase methods. This proved to be true. K_1 did not elute from either a C_{18} or a phenyl column with a mobile phase composed of 90% methanol, with and without derivatized β-cyclodextrins in the mobile phase. Vitamin K_2 did elute from a C_{18} column; however with long run times and excessive tailing. Figure 5.11 shows the retention of K_2 on a phenyl column.

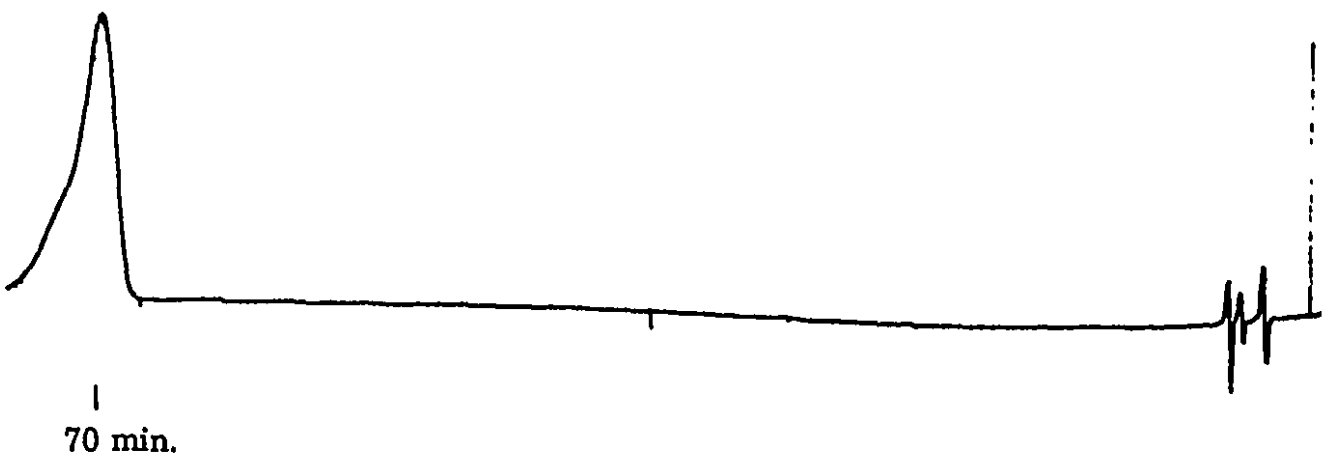


Figure 5.11 Retention of vitamin K_2. Mobile phase was 90:10 methanol:water, 5 mg/ml DM-β-CD.

Cyclodextrin was more successful in assisting in the elution of vitamin K_3. K_3 is not as hydrophobic as its K_1 and K_2 counterparts; therefore it was more likely to partition out of the stationary and form an inclusion complex in the mobile phase. Figure 5.12 shows the effect that cyclodextrin had on the retention of vitamin K_3 on a C_{18} stationary phase. Clearly, vitamin K_3 forms an inclusion complex with dimethyl-β-cyclodextrin as the retention decreased with increasing cyclodextrin concentration. Using the relationship in equation 4.1, the inclusion complex strength was determined to be 60 M. This is a rather weak complex which is why a relatively high concentration (13 mg/ml) of dimethyl-β-cyclodextrin is required to provide a sharp peak with a short retention time. However, the inclusion complex was strong enough so that cyclodextrin can be useful in the analysis of K_3, especially in a complicated matrix such as blood where it must be separated from a large number of possible contaminants. Unfortunately, cyclodextrins failed to have any effect in the reversed-phase analysis of vitamins K_1 and K_2.

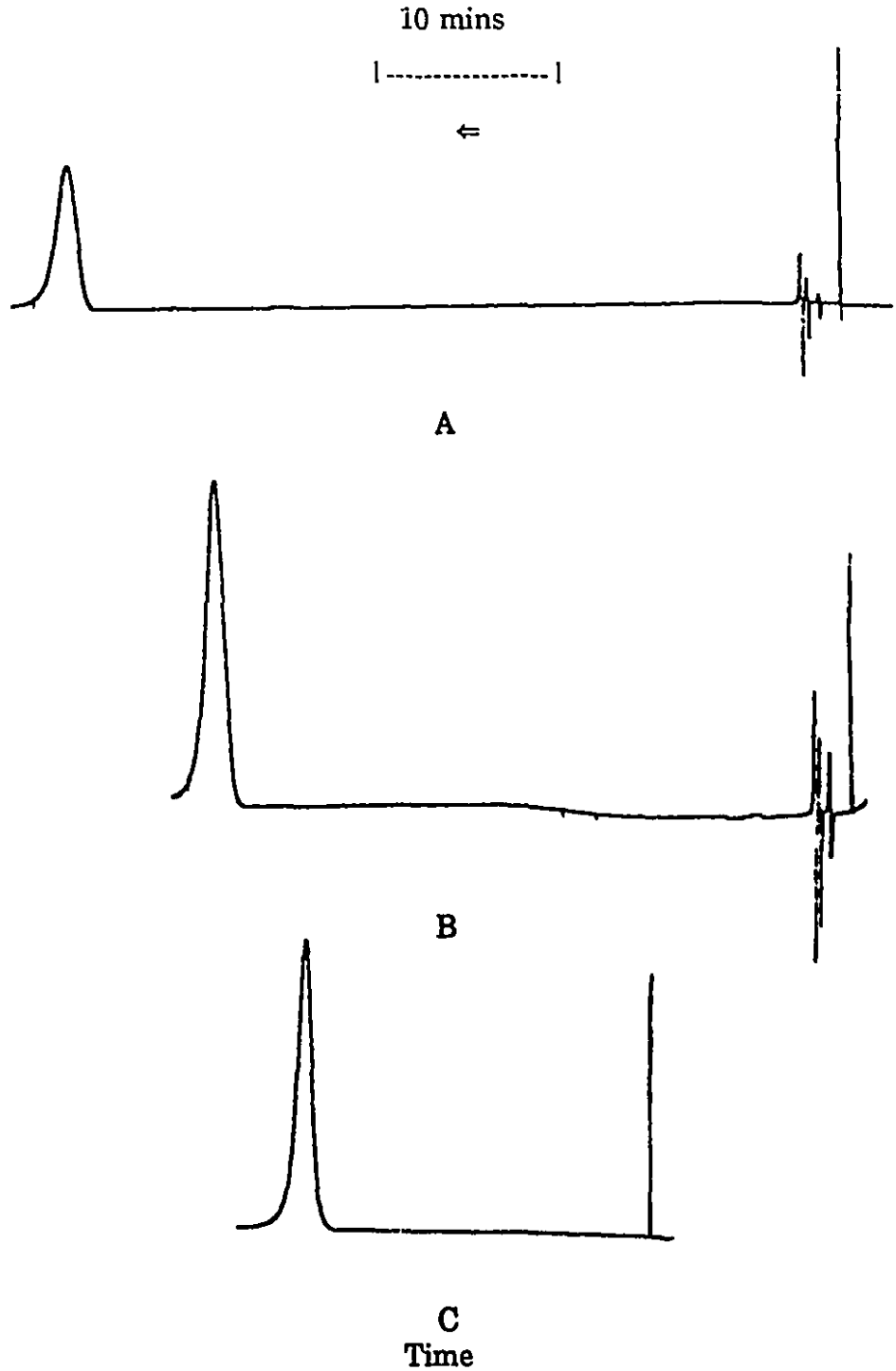


Figure 5.12 Effect of cyclodextrin concentration on retention of vitamin K_3. Mobile phase was 60:40 0.05M KH_2PO_4:methanol with (A) 0 mg/ml; (B) 5 mg/ml; 13 mg/ml DM-β-CD.

5.3.2 Separation of Vitamins by Capillary Electrophoresis

The hydrophobic character of fat-soluble vitamins makes micellar electrokinetic chromatography an obvious choice as a technique for their analysis. The vitamins will solubilze into the hydrophobic interior of an ionic micelle, such as sodium dodecyl sulfate, and be able to migrate electrophoretically. However, as will be shown, MEKC without cyclodextrins was not able to separate any of the vitamins. The addition of cyclodextrins to the running buffer, though, was effective in procuring their separation. Solute separation in micelle-cyclodextrin systems with anionic micelles are based on the neutral vitamins partitioning between the cyclodextrins, which are travelling with the electroosmotic flow towards the detector, and the ionic micelles, which are migrating electrophoretically in the opposite direction. As the electroosmotic flow is typically greater than the electrophoretic velocity, the net migration of the analyte is in the direction of the detector. Inclusion complexation will increase the migration velocity of the solutes through the capillary. This section will discuss the use of CD-MEKC in the separation of fat-soluble vitamins, namely vitamins D_2, D_3, α-, β-, γ-, δ-tocopherol, and the K series.

5.3.2.1 Separation of Vitamins D_2 and D_3

Micellar electrokinetic chromatography, with various cyclodextrins in the buffer, was successful in the separation of vitamins D_2 and D_3. A preliminary separation of the two compounds can be seen in Figure 5.13.

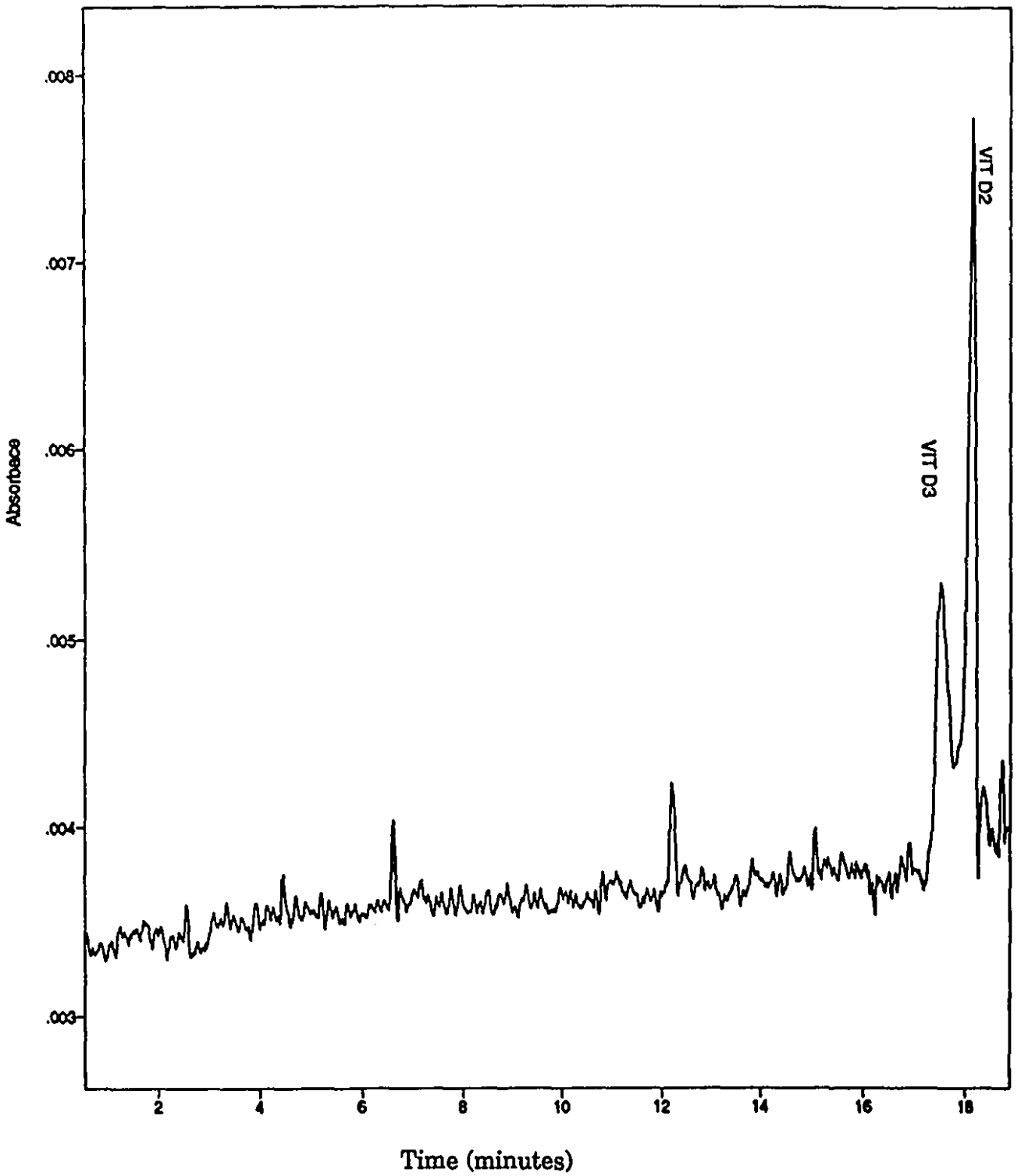


Figure 5.13 Separation of vitamins D_2 and D_3 by CD-MEKC. Conditions: 15 mM phosphate, 20 mM SDS, 25 mM DM-β-CD, pH 7.5, V=15 kV.

As can be seen, this is far from an ideal separation as the peaks are broad and not completely resolved. The excessive band broadening was due to resistance to mass transfer as the vitamins partition in and out of the micelle. To reduce the amount of band broadening, a small amount of ethanol was added to the buffer. The purpose of the addition of an organic modifier is to adjust the partition coefficient of the solutes in the micellar phase so that they are retained less by the micelles and more available to interact with the cyclodextrins in the buffer. The addition of 2% ethanol will have a negligible effect on inclusion complexation.

The addition of 2% ethanol greatly improved the resolution of vitamins D_2 and D_3 as the ensuing reduction in band broadening resulted in baseline separation. The migration times of the solutes did increase, though, as the EOF was reduced due to the presence of the ethanol. Figure 5.14 shows the separation of vitamins D_2 and D_3 using these cyclodextrins in the buffer. Three types of cyclodextrins were examined as buffer additives: β-cyclodextrin, dimethyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin.

The micellar system in the absence of cyclodextrin was not able to distinguish between vitamins D_2 and D_3; however resolution was achieved upon addition of cyclodextrin to the buffer. In the absence of cyclodextrins, the vitamins migrated at the same rate as the micellar flow, indicating that the solutes were not partitioning into the bulk solution. This is hardly surprising due to the vitamins' low water solubility. The micellar flow was estimated with Orange G as a marker.

The best separation was observed with dimethyl-β-cyclodextrin as the buffer additive. Resolution decreased when dimethyl-β-cyclodextrin was replaced with either underivatized β-cyclodextrin or hydroxyethyl-β-cyclodextrin. In the former case, separation was observed; however the limited water solubility of β-cyclodextrin limited its buffer concentration to no more

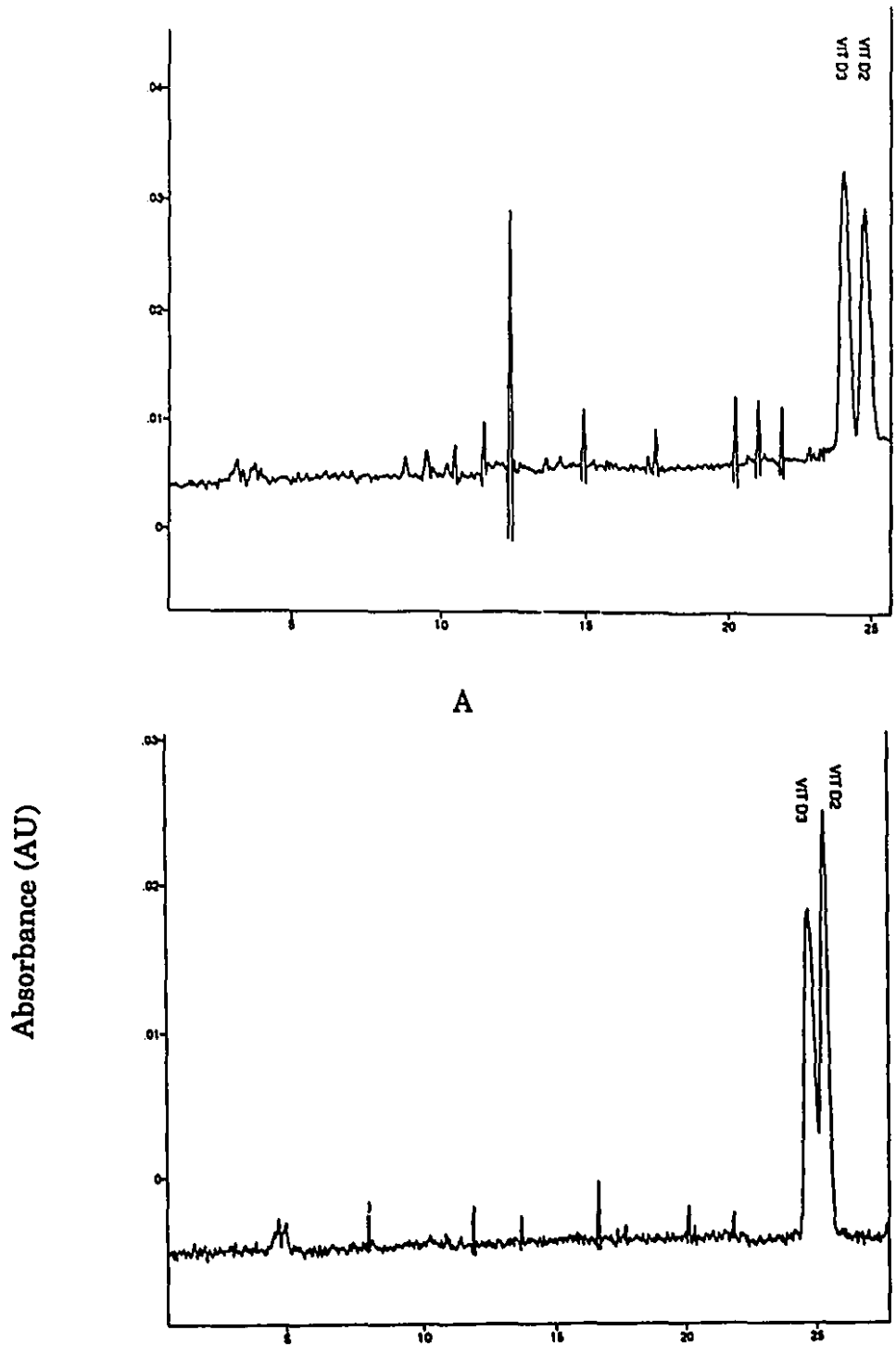


Figure 5.14 Separation of vitamins D_2 and D_3 with various cyclodextrins in the buffer. Conditions: 15 mM phosphate, 20 mM SDS, pH 7.5, V=15 kV, plus (A) 40 mM DM-β-CD; (B) 14 mM β-CD; (C) 40 mM HE-β-CD.

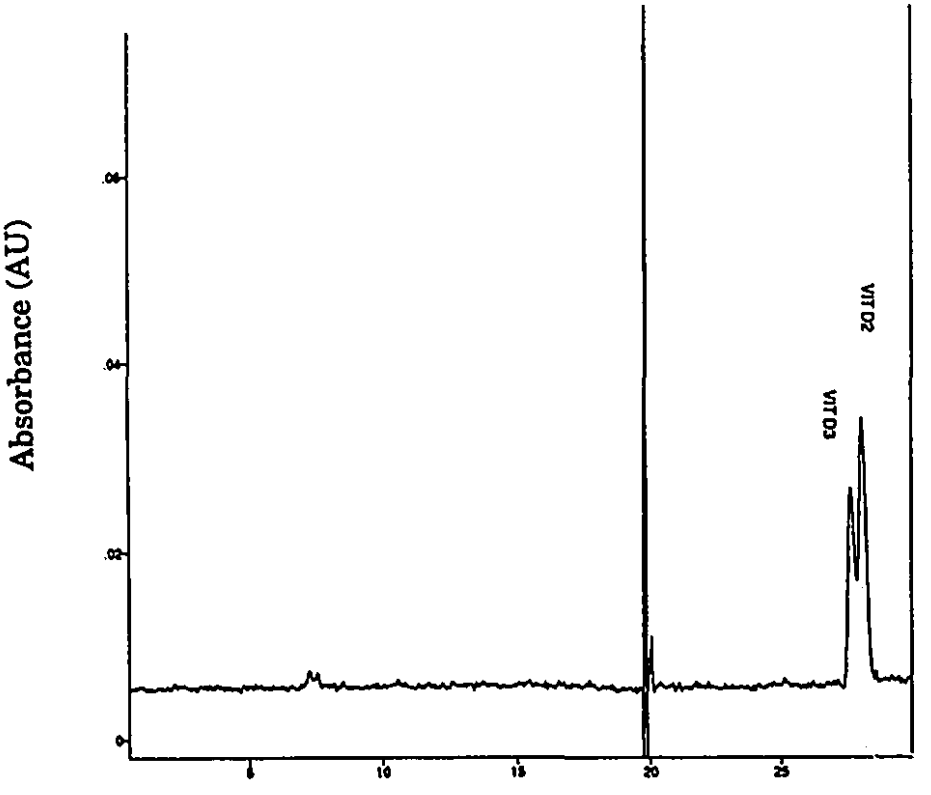


Figure 5.14 continued. Separation of vitamins D_2 and D_3 with various cyclodextrins in the buffer. Conditions: 15 mM phosphate, 20 mM SDS, pH 7.5, V=15 kV, plus (A) 40 mM DM-β-CD; (B) 14 mM β-CD; (C) 40 mM HE-β-CD.

than 14 mM. Comparing hydroxyethyl-β-cyclodextrin to dimethyl-β-cyclodextrin, the methylated derivative gave superior separation as the more hydrophobic cavity formed stronger inclusion complexes than the hydroxyethyl derivative thus allowing more time for separation.

The effect of varying the concentration of all three types of cyclodextrins in the running buffer was studied. Figure 5.15 shows the effect of cyclodextrin concentration on the resolution (R_s) of vitamins D_2 and D_3. The resolution was determined using equation 1.17, where the number of theoretical plates (N) was 97000. N was calculated using equation 5.1.

$$N = 5.54\left(\frac{t_m}{t_w}\right)^2 \qquad (5.1)$$

where t_m is the migration time and t_w is the width of the peak at half height.

Increasing cyclodextrin concentration in the running buffer resulted in improved separation of vitamins D_2 and D_3 with all three types of cyclodextrins studied here. In the absence of cyclodextrin, the two vitamins were not separated. As the cyclodextrin concentration increased, separation improved remarkably with the greatest initial improvement occurring with the underivatized β-cyclodextrin. However, as was mentioned before, the underivatized β-cyclodextrin was not studied above 14 mM due to its limited water solubility. Dimethyl-β-cyclodextrin gave better results than the hydroxyethyl derivative as the stronger inclusion complex formed by DM-β-CD allowed for better resolution of vitamins D_2 and D_3 than HE-β-CD.

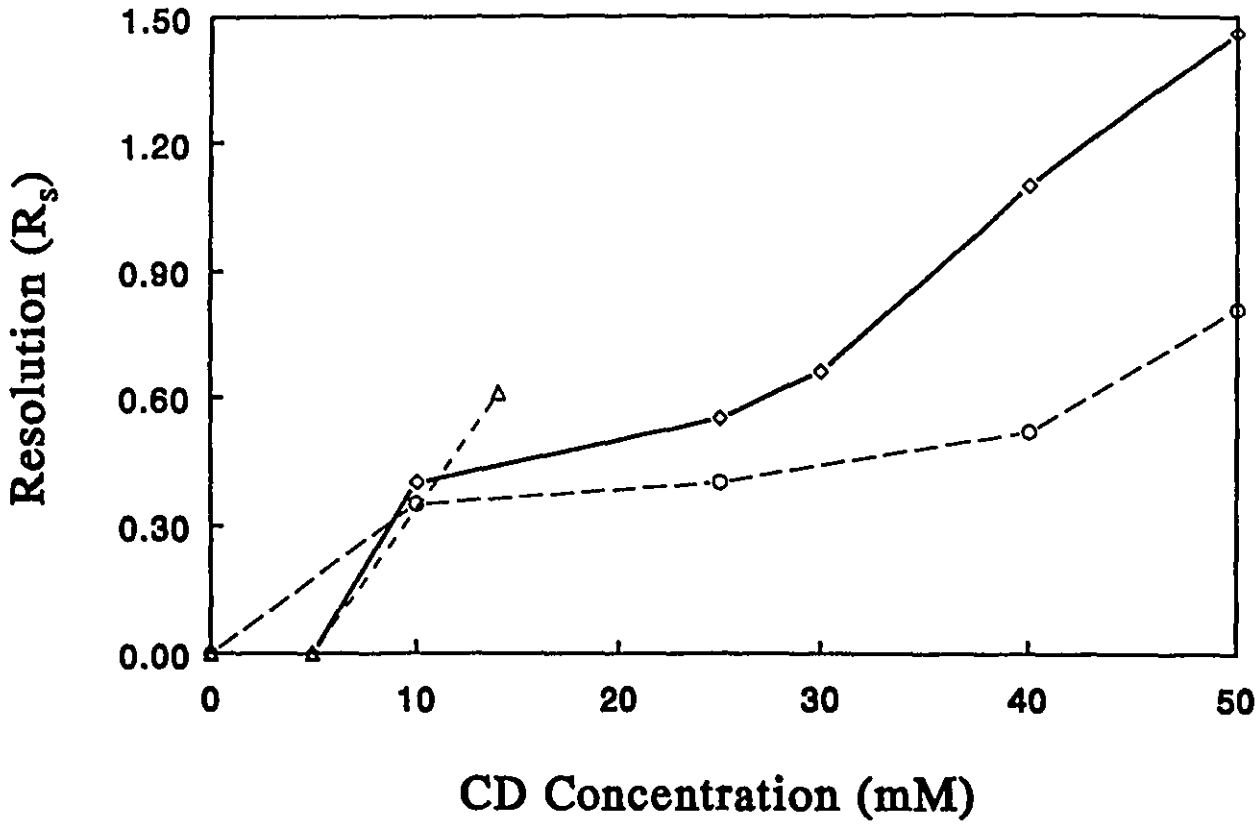


Figure 5.15 Effect of cyclodextrin concentration on resolution of vitamins D_2 and D_3. Conditions: 15 mM phosphate, 20 mM SDS, pH 7.5, V=15 kV, plus the indicated amounts cyclodextrin. ◊=DM-β-CD; △=β-CD; o=HE-β-CD.

5.3.2.2 Separation of E Vitamins

As was mentioned in section 5.3.1.3, the separation of α-, β-, γ-, and δ-tocopherol was generally regarded as difficult to perform due to the similarities in their structures. As was shown in Figure 5.10, cyclodextrins were successful in the partial separation of three of the four vitamers by HPLC. Despite many attempts, resolution of β- and γ-tocopherol was not possible by HPLC; however, a CD-MEKC system was able to resolve all four isomers with DM-β-cyclodextrin in the buffer. Resolution without cyclodextrin was not possible, as can be seen in Figure 5.16.

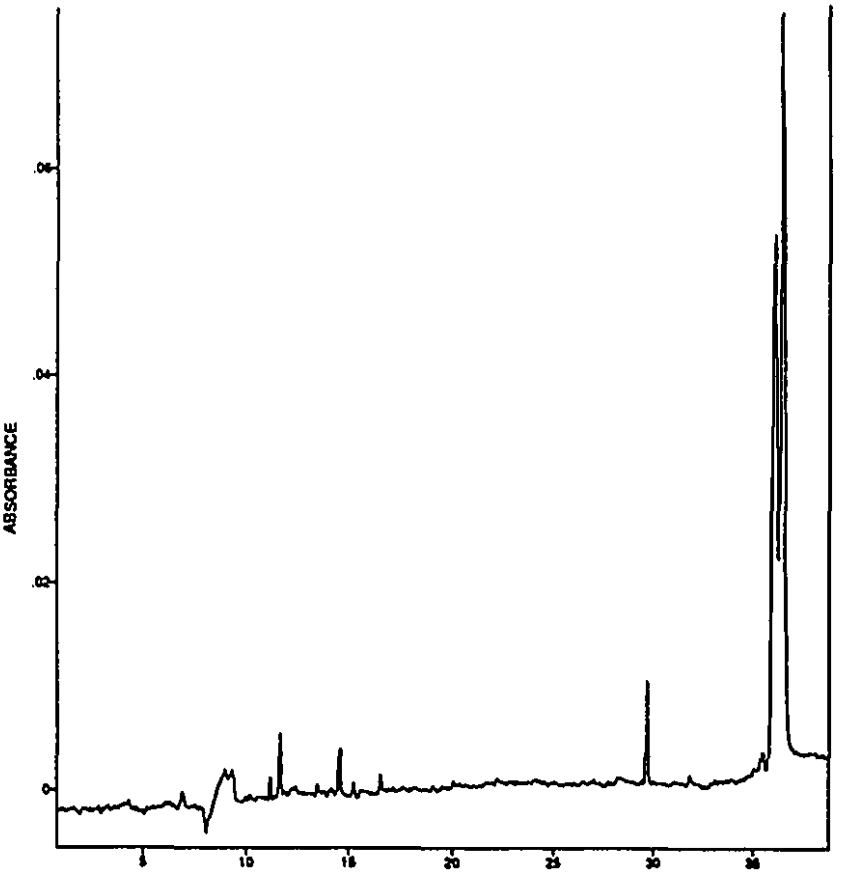


Figure 5.16 Separation of α-, β-, γ-, and δ-tocopherol with no cyclodextrin. Conditions: 20 mM phosphate, 50 mM borate, 30 mM SDS, 2% ethanol, pH 8.0, V=22 kV.

The micellar system alone resulted in only two peaks for the four components. The addition of DM-β-CD to the buffer succeeded in separating all four isomers. Figure 5.17 shows the separation of α-, β-, γ-, and δ-tocopherol by CD-MEKC.

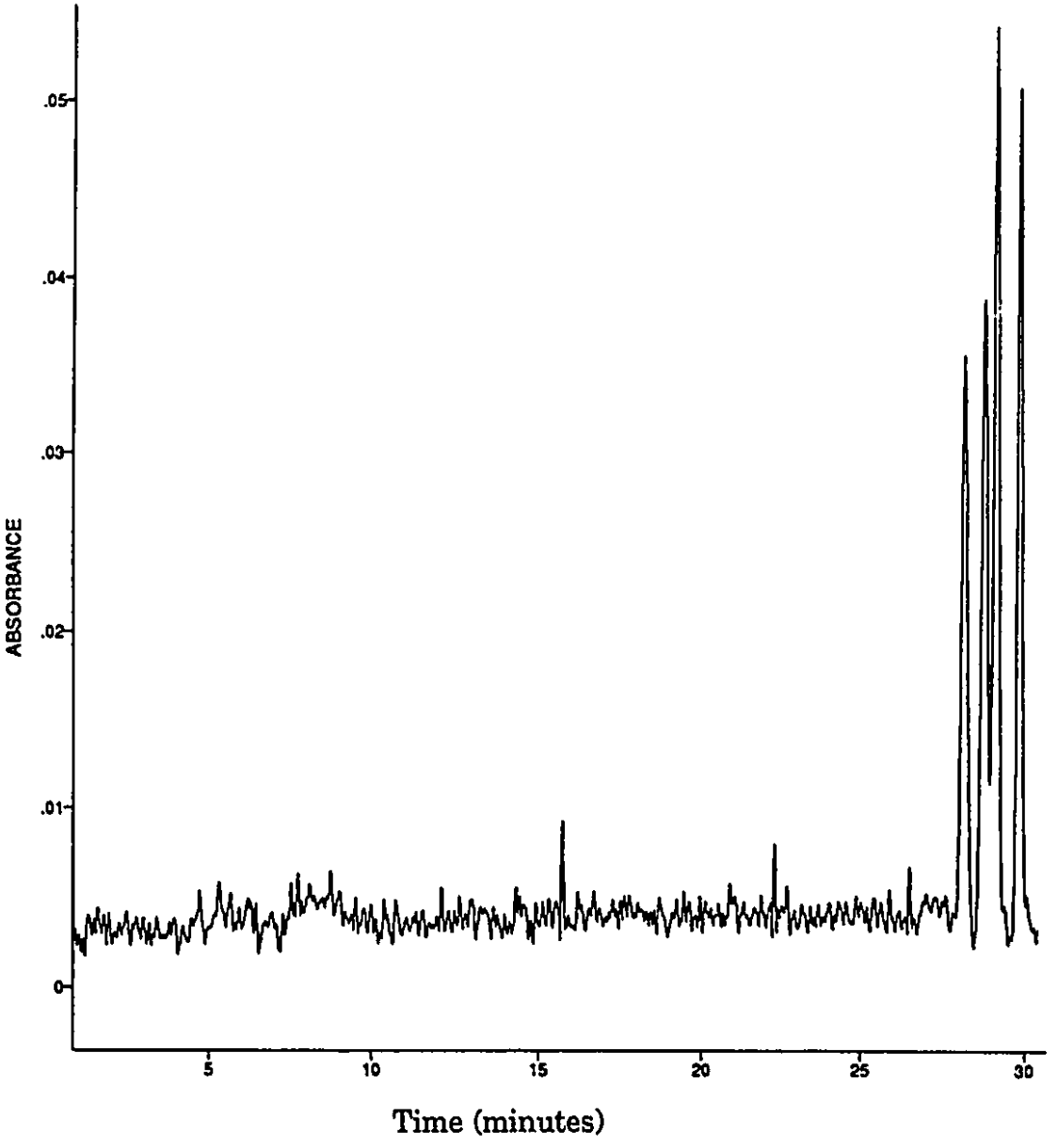


Figure 5.17 Separation of α-, β-, γ-, and δ-tocopherol by CD-MEKC. Conditions: 20 mM phosphate, 50 mM borate, 30 mM SDS, 2% ethanol, pH 8.0, V=22 kV.

The improvement in the separation of the four vitamin E isomers by micellar electrokinetic chromatography over HPLC was due to the greater efficiency observed in capillary electrophoretic techniques. This will be discussed further in section 5.3.3.

The separation of vitamin E (α-tocopherol) from vitamin E acetate was considerably easier by capillary electrophoresis than it was by high performance liquid chromatography (section 5.3.1.3). This was due to the fact that one solute (vitamin E acetate) is charged while the other is neutral. Vitamin E acetate will migrate electrophoretically towards the injection side of the capillary as it partitions in and out of the micelle while α-tocopherol will travel with the electroosmotic flow when not solvated by the micelles. Also to be considered is the fact that the more polar acetate derivative will interact differently with the micelles than vitamin E, which is more hydrophobic.

Figure 5.18 shows the separation of vitamin E from its acetate, with and without dimethyl-β-cyclodextrin in the running buffer. There was good resolution with no cyclodextrin in the buffer for the reasons discussed above. There was only a modest improvement in separation with 25 mM dimethyl-β-cyclodextrin in the buffer. The total migration times for both solutes decreased, an indication that inclusion complexation occurred. The vitamin E complex travelled with the electroosmotic flow towards the detector while the E acetate complex migrated in the opposite direction, but at a slower rate than the uncomplexed molecule due to the increase in charge-to-mass ratio. The almost insignificant increase in separation indicates that the cyclodextrin does not play a important role in the separation of these two compounds by micellar electrokinetic chromatography. Differences in charge and micelle solvation are more important considerations in their separation than inclusion complexation.

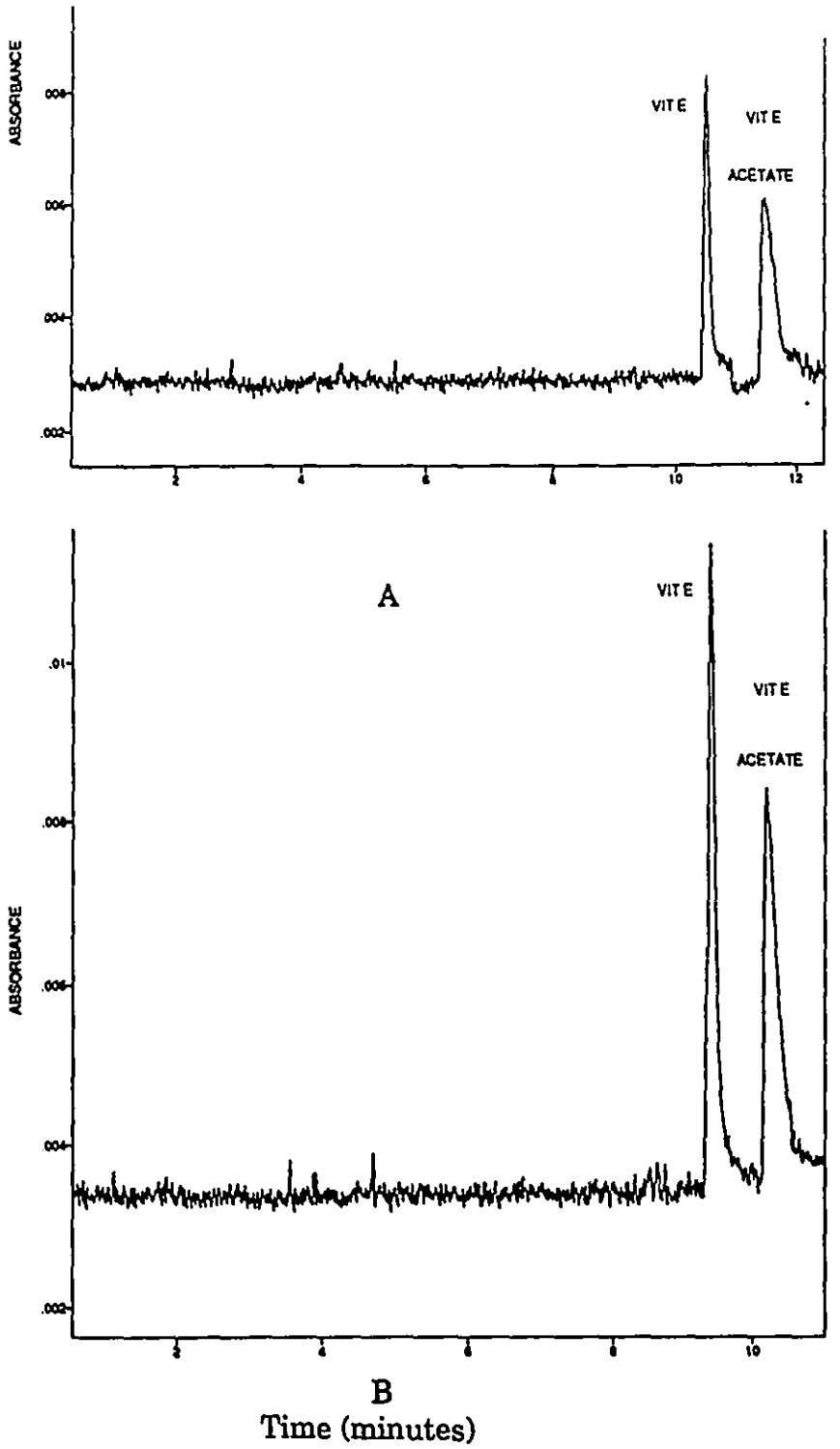
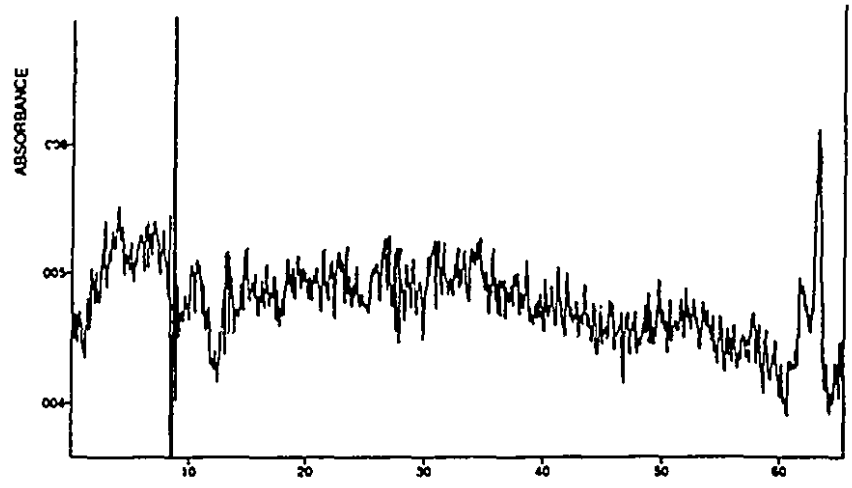


Figure 5.18 Separation of vitamins E and E acetate by MEKC. Conditions: 20 mM borate, 30 mM SDS, pH 8.5, V=15 kV, with (A) 0 mM DM-β-CD; (B) 25 mM DM-β-CD.

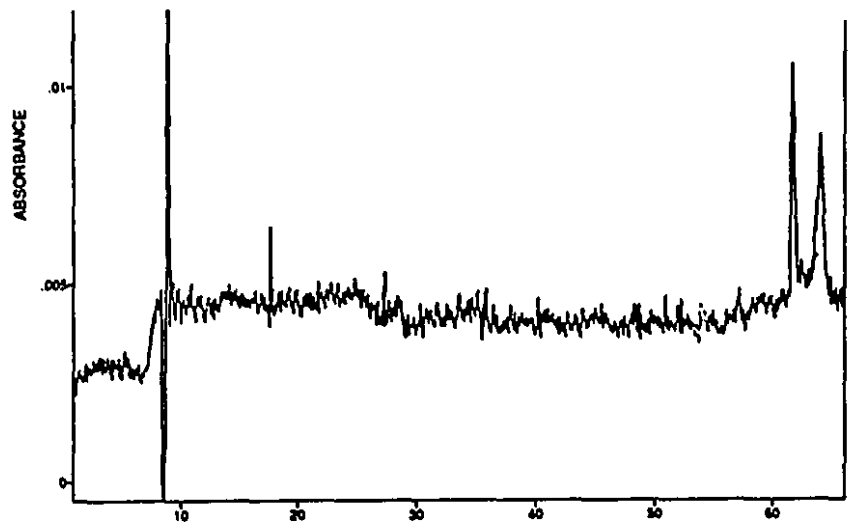
5.3.2.3 Separation of K Vitamins

As was the case with high performance liquid chromatography, MEKC with cyclodextrins was difficult for the analysis of the K vitamins, particularly for the extremely hydrophobic K_1 and K_2. These two molecules interacted very strongly with the micellar pseudo-phase and migrated very closely with the micellar flow (which was estimated by the migration of Orange G). This interaction was so strong that they're migration was barely affected by the presence of cyclodextrin. Figure 5.19 shows the separation of vitamins K_1 and K_2 with no cyclodextrin in the buffer and after the addition of 50 mM dimethyl-β-cyclodextrin. With no cyclodextrin, there is barely any separation of the two solutes. Resolution here was due to differences in solvation inside the micelles. The addition of cyclodextrin procured a slight increase in separation. There was only a slight decrease in migration time as the solutes spent only a small amount of time outside the micelles. This separation required the addition of 2% v/v ethanol in the running buffer to lower the partition coefficient of the solutes in the micelles. One drawback to the analysis of vitamins K_1 and K_2 is the excessively long migration time of over one hour.

Vitamin K_3 is not as hydrophobic as K_1 and K_2, as was seen by its HPLC retention in section 5.3.1.4. Inclusion complexation had a greater effect on K_3 than on K_1 and K_2. The addition of cyclodextrin to the running buffer reduced the migration time of K_3 from 27 minutes to 15 minutes. The migration of vitamin K_3 without modifier and with 40 mM dimethyl-β-cyclodextrin can be seen in Figure 5.20. This compound was not as strongly absorbed by the micelles as K_1 and K_2 therefore it was able to interact favourably with the cyclodextrin in the buffer thus reducing its migration time. Vitamin K_3 did not require the addition of an organic modifier to the buffer to help release it from the micelles.



A



B

Time (minutes)

Figure 5.19. Separation and migration of vitamins K_1 and K_2. Conditions: 25 mM KH_2PO_4, 25 mM borate, 50 mM SDS, 2% ethanol, pH 8.0, V=15 kV, plus (A) 0 mM DM-β-CD; (B) 50 mM DM-β-CD.

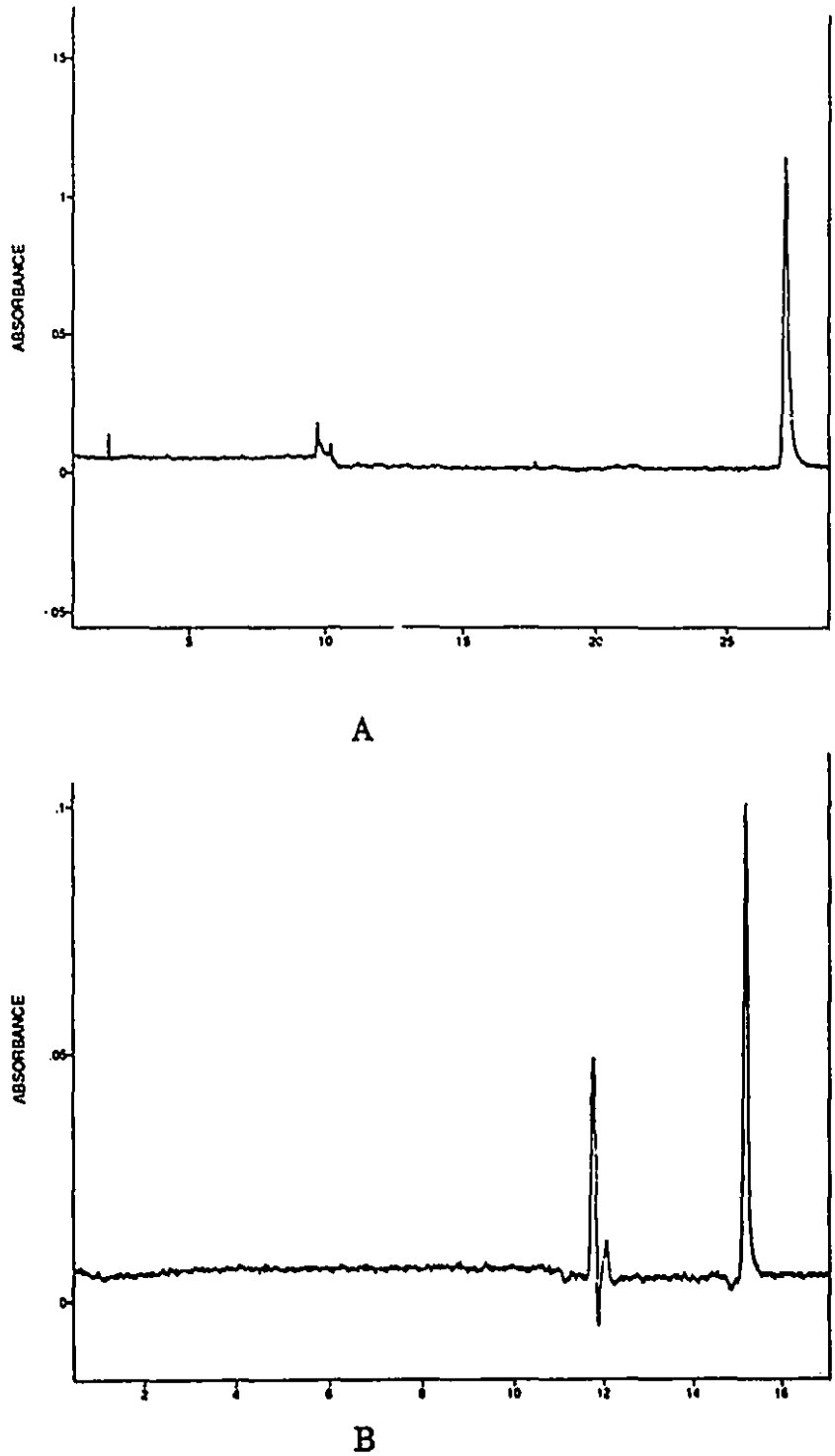


Figure 5.20. Migration of vitamin K_3. Conditions: 25 mM KH_2PO_4, 25 mM borate, 50 mM SDS, pH 8.0, V=17 kV, plus (A) 0 mM DM-β-CD; (B) 40 mM DM-β-CD.

5.3.3 Comparison of Techniques

Certainly, there are some similarities in the separation mechanism for the two methods used in the separation of the fat-soluble vitamins. Both rely on hydrophobic interactions to retain the solutes while using cyclodextrins to decrease retention and provide distinctive interactions that allow for their separation. The differences in the two techniques are evident. In HPLC, the hydrophobic phase, either C_{18} or phenyl, is stationary and generally static. In MEKC, the hydrophobic phase, namely the micelle sodium dodecyl sulphate, migrates in the opposite direction of the desired target (i.e. the detector). As well, micelles are dynamic species which are constantly in equilibrium with the surrounding environment [19]. The surfactant molecules that constitute the micelle are continuously interchanging between the ordered aggregate and the bulk solution. This results in a degree of micellar pseudo-phase heterogeneity that leads to band broadening [20].

Another major difference between the two techniques is the column efficiency, or number of theoretical plates. The sources of band broadening for both techniques were discussed in Chapter 1. To reiterate, band broadening sources in HPLC include eddy diffusion, resistance to mass transfer in the mobile and stationary phases, longitudinal diffusion, stagnant mobile phase mass transfer, and extracolumn broadening. The absence of a packing in capillary electrophoresis eliminates these sources of broadening, with the exception of longitudinal diffusion and extracolumn causes. Capillary electrophoresis does suffer from its own unique sources of band broadening, namely temperature gradients due to Joule heating, adsorption on the capillary walls, and electromigrative dispersion. The addition of the micellar pseudo-phase to the running buffer creates additional band broadening sources such as intermicellar mass transfer, differences in sorption-desorption kinetics of the solutes entering and exiting the micelles, and micellar heterogeneity.

In comparing the results obtained for the fat-soluble vitamins by HPLC and MEKC, it can be said the use of the more efficient capillary electrophoretic technique gave mixed results. Both techniques gave reasonably good results in the separation of vitamins D_2 and D_3. At their optimal separation conditions, the HPLC method gave slightly better R_s values than CE (1.75 vs 1.50) with dimethyl-β-cyclodextrin as additive; nonetheless, both techniques proved suitable for the analysis of these two compounds. The presence of cyclodextrin was not imperative for the high performance liquid chromatographic method; however separation and retention improved greatly upon addition of cyclodextrin to the mobile phase. For the micellar electrokinetic chromatographic technique, cyclodextrins were essential in the successful separation of vitamins D_2 and D_3 as the ionic micelle alone was not able to differentiate between the two molecules.

Undoubtedly, MEKC gave a better separation of vitamins E and E acetate than HPLC. This separation takes advantage of the fact that one solute migrates electrophoretically and the other one does not. Also, due to its reduced hydrophobic character, E acetate is not as strongly retained by the micelles as α-tocopherol. This separation did not require the presence of cyclodextrin to the buffer. The subsequent addition of the inclusion complexation interaction proved to offer little improvement in their separation. This contrasted the HPLC method, where cyclodextrin improved their separation. This was because HPLC could not take advantage of the solutes' differences in charge the way electrophoresis can.

Micellar electrokinetic chromatography proved to be better than high performance liquid chromatography in the separation of the four isomers of vitamin E. It had been reported than a β-cyclodextrin stationary phase was able to separate α-, β-, γ-, and δ-tocopherol [9]; hence it was known that all four isomers exhibited different inclusion complex strengths with β-cyclodextrin.

HPLC could not capitalize on the differences in inclusion complex strength with cyclodextrins as mobile phase additives, as was shown in Section 5.3.1.3. Band broadening resulting from the excessive run times made the resolution of β- and γ-tocopherol futile. Of particular consequence was the broadening due to mass transfer between the extremely hydrophobic solutes and the stationary phase. The absence of a packing implies that this source of band broadening is not observed in MEKC; therefore the peaks were sharper and better resolved. MEKC suffers from its own distinctive sources of band broadening, specifically resistance to mass transfer in and out of the micelle. However, the broadening in MEKC was not as significant as in HPLC, evident by the separation of the four vitamin E isomers. The hydrophobic D vitamins also suffered from this band broadening in HPLC; however the two molecules were dissimilar enough to ensure their complete resolution by differences in inclusion complex strength with cyclodextrin. In the separation of β- and γ-tocopherol by HPLC, the difference in inclusion complex strength was not enough for their resolution.

Both high performance liquid chromatography and micellar electrokinetic chromatography gave unimpressive results for the vitamin K series. In both cases, the high hydrophobic character of the solutes made them difficult to remove from the hydrophobic phases. It was shown that vitamin K_3 formed inclusion complexes and was able to be analyzed using either HPLC or MEKC in a reasonable amount of time. The results were different for vitamins K_1 and K_2. Cyclodextrin had little effect on their run times using both techniques as they were strongly retained by the hydrophobic phases. For HPLC, even the use of a less hydrophobic phase did not improve their analysis results. For MEKC, better results may be obtained with a less hydrophobic micellar phase. While not attempted, sodium cholate or other bile salts could possibly be used as the micellar phase. However, bile salts form strong

inclusion complexes with cyclodextrins [21]. Separation would be compromised as the solutes would have to compete with the uncomplexed surfactants for inclusion complexation in the cyclodextrin cavity.

5.4 CONCLUSIONS

It has been demonstrated that using cyclodextrins in reversed-phase high performance liquid chromatography and micellar electrokinetic chromatography proved to be useful in the separation of fat-soluble vitamins. The use of MEKC over HPLC gave either comparable or improved separation of these extremely hydrophobic solutes. Both techniques were successful in the separation of vitamins D_2 and D_3. Dimethyl-β-cyclodextrin provided the best separation of these two compounds. Generally, separation increased in both techniques with increasing cyclodextrin concentration.

The addition of cyclodextrin to the mobile phase aided in the separation of vitamins E and E acetate by HPLC with a phenyl column. The addition of cyclodextrin to the running buffer was not necessary in their separation by MEKC; this could be achieved solely on the basis of charge. The addition of cyclodextrin to the buffer offered little improvement in separation. In the separation of α-, β-, γ-, δ-tocopherol, cyclodextrins were not able to separate β- and γ- tocopherol by HPLC; however they were successful in separating the other isomers. The use of cyclodextrin in MEKC procured the resolution of all four isomers.

Both techniques failed to provide suitable operating conditions for the analysis of vitamins K_1 and K_2, even with the aid of cyclodextrins. The formation of inclusion complexes did assist in reducing the run times of vitamin K_3 by both reversed-phase HPLC and MEKC. Both techniques would be suitable in the analysis of K_3 from a complex matrix.

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CHAPTER 6

CAPILLARY ELECTROPHORETIC SEPARATION OF TRICYCLIC ANTIDEPRESSANTS WITH CHARGED CARBOXYMETHYL-β-CYCLODEXTRIN

6.1 INTRODUCTION

As was discussed in chapter 2, cyclodextrins have been employed frequently as buffer additives in capillary electrophoresis for the analysis of both charged and uncharged molecules. Neutral cyclodextrins can be used in the separation of charged molecules as they migrate electrophoretically while the cyclodextrin migrates with the electroosmotic flow. However, for the analysis of uncharged or similarly structured charged molecules, neutral cyclodextrins must be used in conjunction with a charged additive, such as ionic micelles. This technique is called micellar electrokinetic chromatography (MEKC). The solute molecules partition between the micelle phase, which migrates electrophoretically, and cyclodextrin, which migrates with the electroosmotic flow. One complication with this system is that the micellar pseudophase contributes significantly to band broadening [1]. This band broadening

has been attributed to resistance to intermicelle mass transfer, radial thermal gradients within the micellar system, and micellar heterogeneity.

The need for micelles can be eliminated by using charged cyclodextrins in the buffer. Cyclodextrins in which the rim groups have been replaced with charged functional groups can be used as a carrier for uncharged solutes or similarly-structured charged molecules without the need for micelles or other complexing agents. Cyclodextrin's ability to migrate electrophoretically makes it suitable for the separation of both neutral and charged molecules. In addition, the charged functional groups can provide electrostatic interactions between an ionic solute and cyclodextrin [2]. Ionic cyclodextrins, such as carboxymethyl-β-cyclodextrin, will attract solutes with opposite charge and repulse ones with a similar charge, allowing for yet another means for cyclodextrins to distinguish between solutes.

To date, the majority of research carried out using ionic cyclodextrins has focused on chiral separations [3-6]. However, charged cyclodextrins have also proven to be useful for non-chiral separations, such as the separation of the positional isomers of nitrobenzyl alcohol [7] and xylidine [8]. The use of charged cyclodextrins in capillary electrophoresis has not been fully explored and could prove to be a suitable alternative to the CD-MEKC system.

This chapter describes the use of an ionic cyclodextrin, namely carboxymethyl-β-cyclodextrin (CM-β-CD), as a buffer additive in the separation of a series of tricyclic antidepressants. Tricyclic antidepressants are a class of highly lipid-soluble compounds used extensively in the treatment of depression and other related illnesses. These drugs are believed to work by blocking the re-uptake of neurotransmitters by the brain cells, thus increasing the amounts of available transmitters, specifically serotonin and norepinephrine [9].

There is sufficient evidence to believe tricyclic antidepressants form strong enough inclusion complexes to make possible their separation and

analysis by capillary electrophoresis. Brewster and co-workers [10] established that this class of compounds form strong inclusion complexes by studying the increase in solubility of carbamazepine in the presence of a variety of derivatized β-cyclodextrins while Piperaki and co-workers [11] separated some tricyclic antidepressants by HPLC using a β-cyclodextrin stationary phase.

The aim of this work was to separate a series of nine tricyclic antidepressants, all having similar structures, with charged cyclodextrin as the only buffer complexing agent. As will be discussed, carboxymethyl-β-cyclodextrin had limited success in the separation of the antidepressants studied here. This chapter will discuss the separation of these antidepressants as well as look at the effect of varying operating conditions such as pH and cyclodextrin concentration. The discussion will also include a comparison of separation performance when a variety of capillary coatings are used to reduce the electroosmotic flow. Finally, the successful separation of all nine antidepressants using agents in addition to carboxymethyl-β-cyclodextrin will be discussed.

6.2 INSTRUMENTAL

6.2.1 Apparatus

The capillary electrophoretic apparatus was described in section 5.2.1. Separations were performed on fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) of 50 μm I.D. and 375 μm O.D. If necessary, these capillaries were coated as described in section 6.2.4. Electropherograms were acquired with the Waters System Interface Module (Millipore Corp. Milford, Mass, USA) and then processed on the Waters Maxima 820 Chromatography Workstation. A diagram of the CE system used can be found in Figure 1.3.

6.2.2 Chemicals

Tricyclic antidepressants, of analytical grade or better, were obtained as gifts from Lilly, Merck Frosst, or purchased from Sigma (St. Louis, MO, USA). Ultra pure sodium dodecyl sulfate (SDS) and boric acid were obtained from ICN Biochemicals (Montreal, QC, Canada). Sodium tetraborate (Borax), 3-(trimethoxysilyl)propylmethacrylate (MAPS), octyltrimethoxysilane, octadecyl-trimethoxysilane, and isobutyltrimethoxysilane were obtained from Aldrich (Milwaukee, WI, USA). Sodium hydroxide, glacial acetic acid, and hydrochloric acid were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ultra pure acrylamide, ammonium persulfate, and N,N,N',N'-tetramethylethylenediamine (TEMED) were purchased from Biorad (Mississauga, ON, Canada). Carboxy-methyl-β-cyclodextrin was purchased from Cyclodextrin Technologies Development (Gainesville, FL, USA). Potassium phosphate was obtained from A&C Chemicals (Montreal, QC, Canada). Citric acid was purchased from Anachemia (Montreal, QC, Canada). Water was doubly distilled and deionized.

6.2.3 CE Procedures

The capillaries had a total length of 70 cm and a separation length of 55 cm and were conditioned prior to use for 10 minutes with 1N NaOH. Coated capillaries were rinsed between runs for two minutes with distilled water. The detection wavelength was 254 nm. Unless otherwise noted, an applied voltage of 22 kV was used for all separations. The pH of the running buffer was adjusted by the addition of appropriate quantities of solutions of boric acid, hydrochloric acid or sodium hydroxide. All buffers were filtered through a 0.45 μm membrane and then degassed prior to use. Samples were dissolved in the running buffer, degassed, and injected hydrodynamically at a height of 15 cm; the injection time was 8-10 seconds.

6.2.4 Preparation of Coatings

The capillaries were coated by the method developed by Hjertén [12], with some modifications. The bare capillary was first conditioned with 1N NaOH for 15 minutes and then rinsed for 5 minutes with water. Next, the capillary was treated for one hour, using house vacuum, with a solution containing 30 µL of 3-(trimethoxysilyl)propyl methacrylate (MAPS) in 1 mL of 1:1 (v/v) acetic acid and water. The excess MAPS was rinsed with water. For capillaries that were coated further with polyacrylamide, the polymerizing solution was prepared by adding 10 µL of TEMED (polymerization initiator) to 10 mL of a degassed 4% (w/v) aqueous acrylamide solution, followed by 10 µL of 10% (w/v) ammonium persulfate (creates TEMED radical). The capillary was filled with the solution and the polymerization reaction was allowed to proceed for one hour at which point the excess polyacrylamide was flushed and the capillary was rinsed with water.

6.3 DISCUSSION

The chemical structures of the nine tricyclic antidepressants under investigation can be found in Figure 6.1. The compounds can be divided into two groups. The first group is comprised of compounds with a C-N linkage between the central seven-membered ring and the alkyl side chain extending from the ring. This group is made up of carbamazepine (CAR), trimipramine (TRI), opipramol (OPI), imipramine (IMI), desipramine (DES), and clomipramine (CLO). The second group consists of molecules with a C-C or C=C linkage between the side chain and the centre ring. This group comprises of amitriptyline (AMI), nortriptyline (NOR), and protriptyline (PRO).

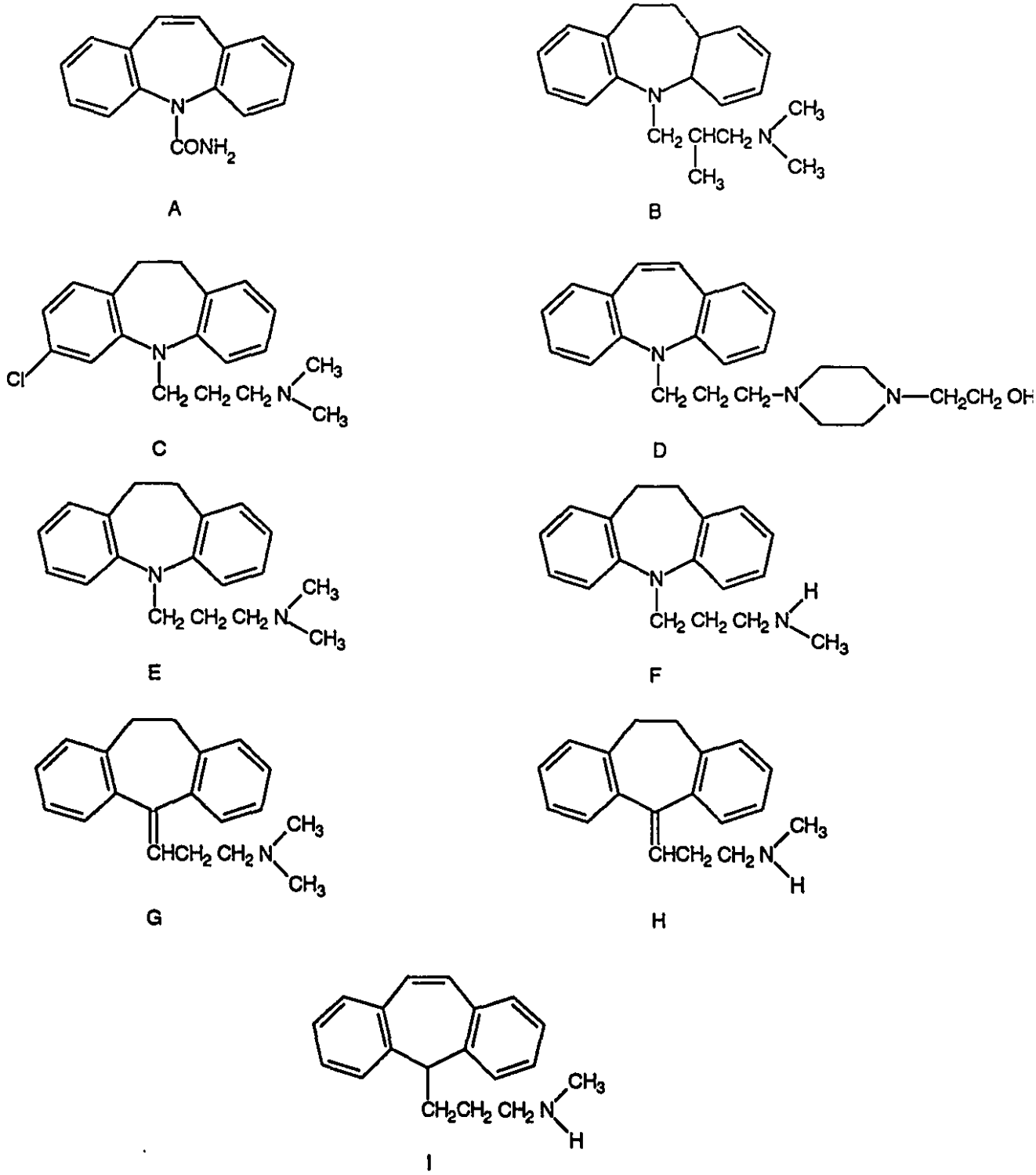

Figure 6.1 Structure of antidepressants. **A** Carbamazepine (CAR); **B** Trimipramine (TRI); **C** Clomipramine (CLO); **D** Opipramol (OPI); **E** Imipramine (IMI); **F** Desipramine (DES); **G** Amitriptyline (AMI); **H** Nortriptyline (NOR); **I** Protriptyline (PRO).

6.3.1 Preliminary Separations Using Carboxymethyl-β-cyclodextrin

As was previously noted, the preliminary aim of this work was to separate nine similarly-structured tricyclic antidepressants with carboxymethyl-β-cyclodextrin as the only complexing agent in the running buffer. Initially, attempts were made to separate all nine compounds on a bare silica capillary under normal operating conditions. The pH of the running buffer for this separation was important in order to keep the cyclodextrin charged. The pH had to be kept above 4.5 as the degree of ionization of carboxymethyl-β-cyclodextrin is dependant on the pH. The pKa of the carboxyl groups on CM-β-CD was quite low and had been determined by electrochemical quartz crystal microbalance to be approximately 4.15 [13]. A pH below this value would mean that the cyclodextrin would be predominantly in its protonated form. The loss of charge would deter its migration and result in a loss in separation. The pH was also an important consideration for the antidepressants. At a pH below 9, the amine groups on the antidepressants become fully protonated and do migrate electrophoretically. However, Salomon and co-workers [14] confirmed that they cannot be separated by capillary electrophoresis below pH 9 as their charge/mass ratios are too similar for separation.

The suitability of this system was tested on mixtures containing two of the antidepressants. Carboxymethyl-β-cyclodextrin was nominally successful in separating a pair of antidepressants, one from each of the structural groups. Figure 6.2 shows a preliminary separation of two antidepressants, nortriptyline and desipramine on a bare silica capillary.

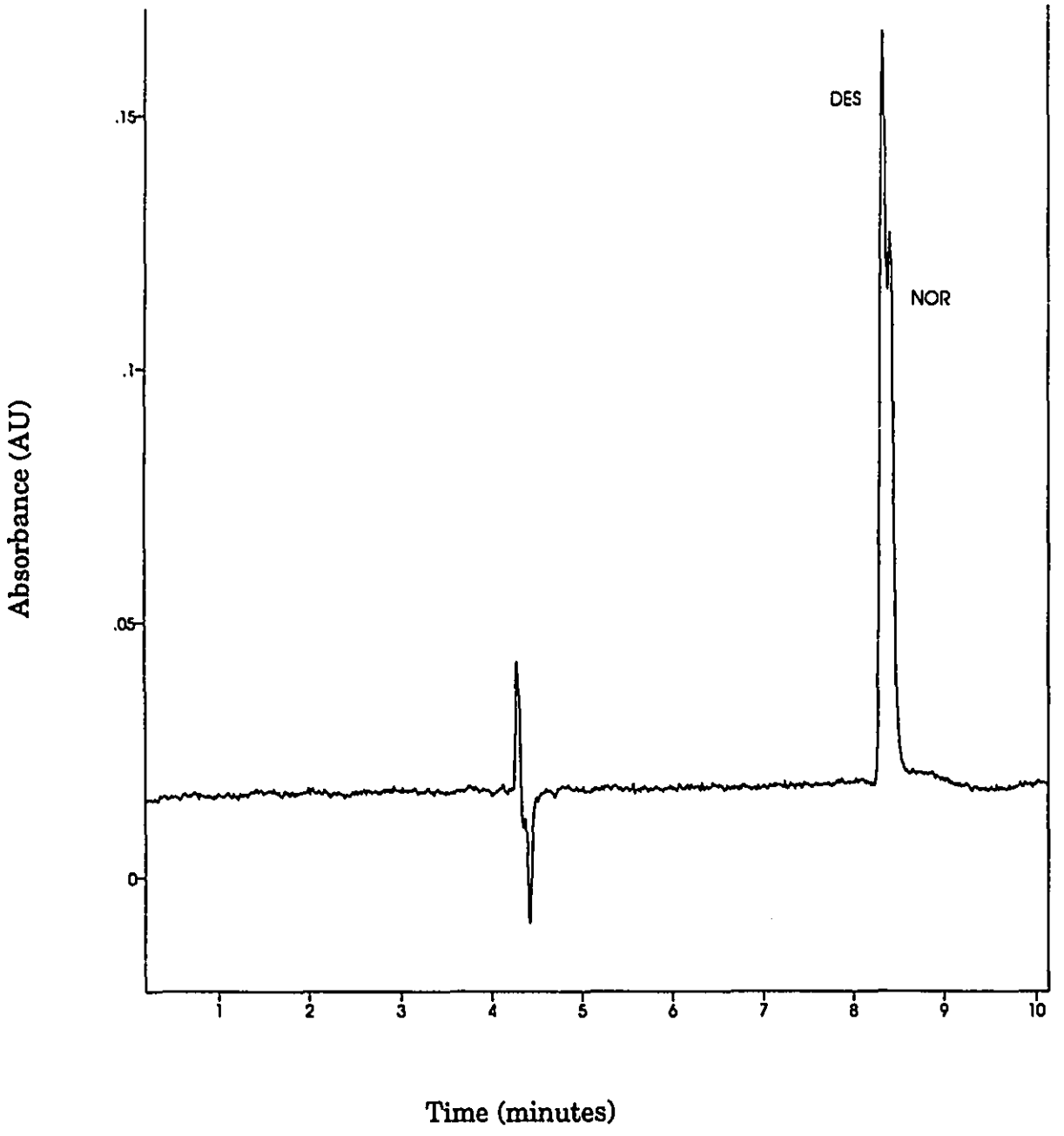

Figure 6.2 Separation of nortriptyline and desipramine. Buffer: 25 mM phosphate, 25 mM borate, 20 mM CM-β-CD, pH=6.3; V=15 kV.

Although the separation of these antidepressants was poor, these preliminary results on a bare silica capillary were promising enough to demonstrate that cyclodextrin was able to distinguish between the two molecules. The minimal resolution was a result of the high electroosmotic flow. The EOF, with a migration time of approximately four minutes (for a rate of 13.75 cm/minute), was too fast to allow the cyclodextrin to achieve complete resolution. This became even more evident when the system was used to attempt the separation of a mixture of all nine antidepressants. Under these conditions, carboxymethyl-β-cyclodextrin was unsuccessful in resolving these compounds due to the overlapping bands from the closely eluting solutes. This separation can be seen in Figure 6.3.

The velocity of the electroosmotic flow is an important parameter in capillary electrophoresis. In this work, separation occurred by differences in complexation with the anionic cyclodextrin which retarded the solute's net migration. The inclusion complex travelled electrophoretically in the opposite direction to the EOF and the rapid EOF hindered inclusion complexation and hence adversely affected separation. In order to improve separation, the EOF had to be slowed or eliminated. Several options were considered:

(i) Increase the ionic strength of the buffer to decrease the electroosmotic flow [15]. An increase in the ionic strength of the running buffer decreases the zeta potential, which can be given as [16]:

$$\zeta = \frac{4\pi\delta e}{\epsilon} \qquad (6.1)$$

where e is the total excess charge in a given area, δ is the double layer thickness (Debye ionic radius), and ε is the buffer's dielectric constant. The Debye ionic radius can be expressed as:

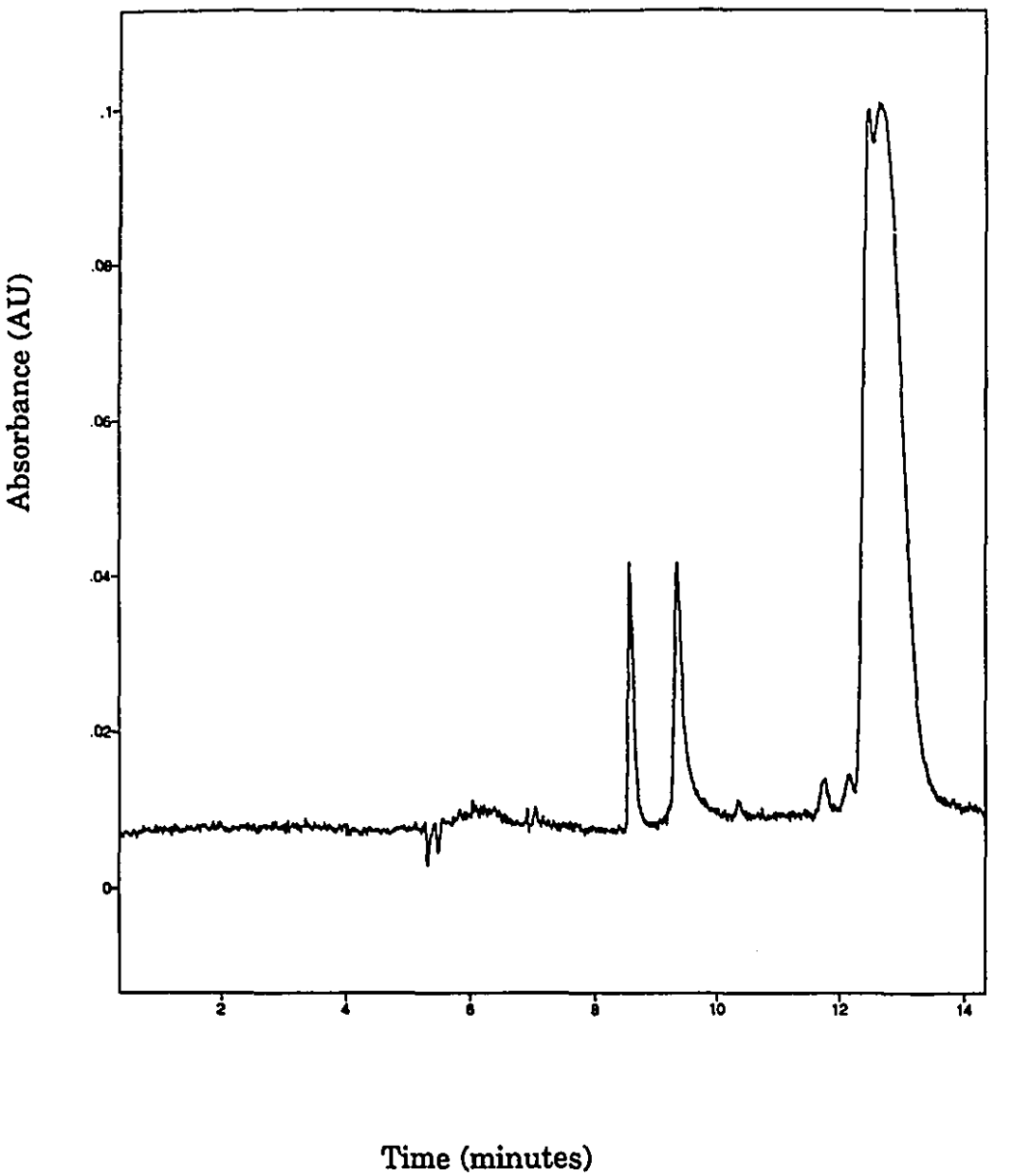

Figure 6.3 Separation of nine antidepressants on a bare silica capillary. Conditions same as Figure 6.2.

$$\delta = \frac{1}{3\times10^{7}|Z|C^{1/2}} \qquad (6.2)$$

where Z is the charge and C is the ionic strength. Therefore, as the ionic strength increases, the zeta potential decreases thus reducing the electroosmotic flow. Increasing ionic strength, however, was not a feasible strategy as the resulting increase in current would increase joule heating and band broadening.

(ii) Decrease the pH. This was ruled out as decreasing the pH would decrease the degree of ionization of the cyclodextrin, adversely affecting electrophoretic migration and separation.

(iii) Coat the inside of the capillary. This technique was chosen for reducing the electroosmotic flow.

Coating the inside of the capillary serves two functions. The first is that it reduces or eliminates the EOF by covering the ionized silanol groups thereby eliminating the compact layer of cationic buffer molecules responsible for the EOF. The second function of the capillary coating, which is much less of a concern for this work, is to reduce cationic solute adsorption on the capillary wall [12].

It was originally hypothesized that the elimination of the electroosmotic flow would procure the desired separation of all nine compounds. The inside of the bare silica capillary was coated with 4% polyacrylamide, as described in section 6.2.4, and the polarity of the applied voltage was reversed so the cyclodextrin could transport the solute to the detector. Methanol and benzyl alcohol, injected onto the coated capillary as EOF markers, did not elute after 70 minutes, indicating no significant electroosmotic flow.

The polyacrylamide coated capillary did not give satisfactory results. The peaks were too broad due to the long migration times. The separation of

desipramine and nortriptyline on a 4% polyacrylamide capillary can be seen in Figure 6.4.

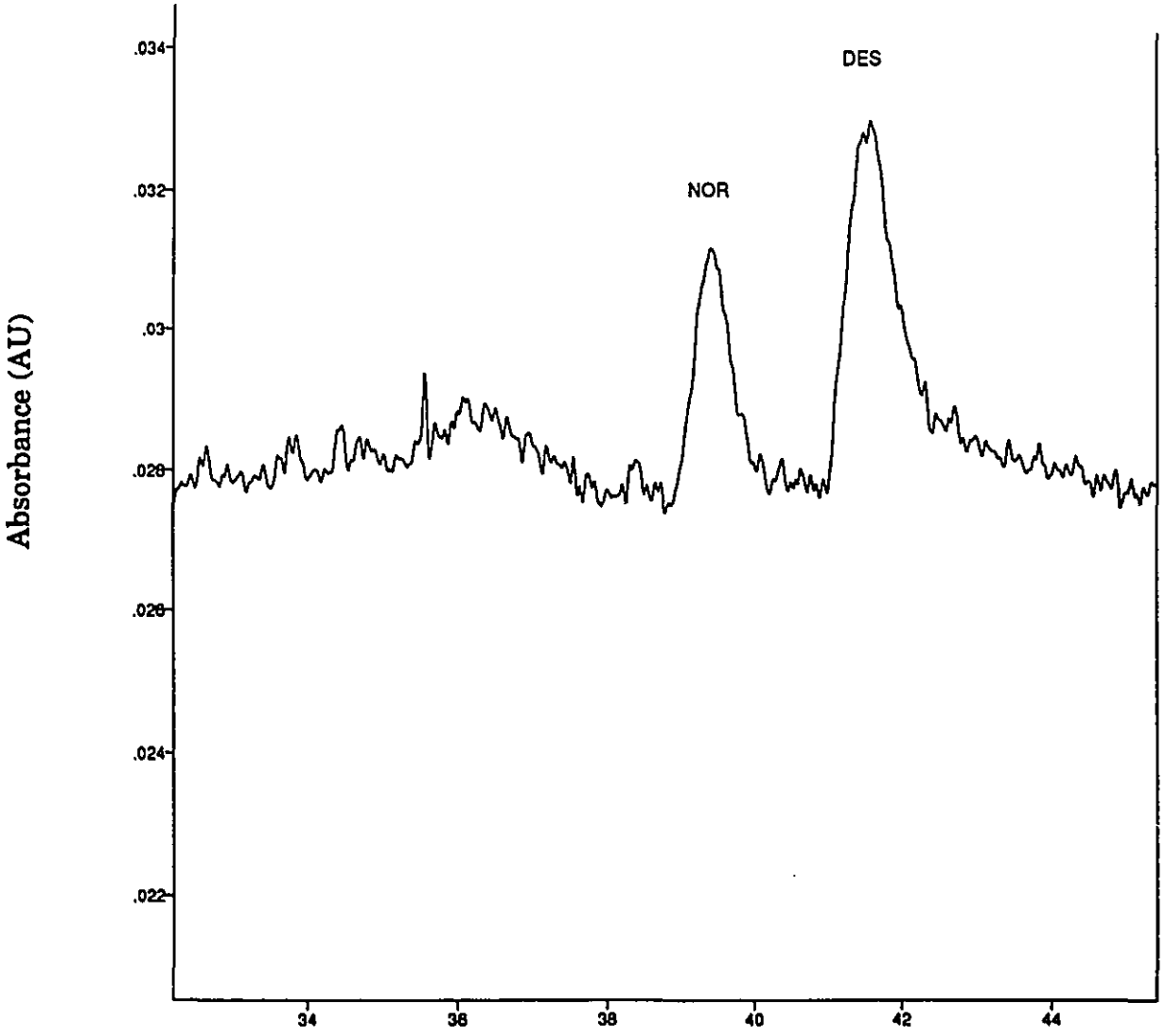

Figure 6.4 Separation of desipramine and nortriptyline on a 4% polyacrylamide capillary. Conditions: 10 mM phosphate, 10 mM CM-β-CD, pH 6.25.

The elimination of the electroosmotic flow resulted in excessively long run times as the cyclodextrin was the only solute carrier. It was decided that a system with an EOF between the high rate on bare silica and the very negligible rate on polyacrylamide-coated capillaries would be necessary. That is, reduce but not eliminate the electroosmotic flow. Dougherty and co-workers [17] reported that capillary coatings with alkyl functionalities, whose preparation methods were proprietary and hence not reported, were useful in reducing the EOF but not eliminating it. It was decided to attempt to use a capillary coated with only 3-(trimethoxysilyl)propylmethacrylate (MAPS) as the strategy in reducing the EOF. The MAPS coating was prepared by following the acrylamide coating procedure outlined in the experimental section, except that the acrylamide polymerization step was omitted. The reaction for the coating process of MAPS onto the fused silica surface (Si-OH) was as follows [12]:

$$Si\text{-}OH + (CH_3O)_3\text{-}Si\text{-}R \longrightarrow Si\text{-}O\text{-}Si(CH_3O)_2\text{-}R \qquad (6.3)$$

This coating process resulted in a capillary that reduced, but not eliminated, the electroosmotic flow.

The polarity of the applied voltage was inverted once again so that a positive potential was applied to the injection end of the capillary. The MAPS capillary had an EOF of approximately 26 minutes (rate of 2.1 cm/minute) at an applied voltage of 25 kV with a buffer comprised of 5 mM KH_2PO_4, 10 mM CM-β-CD, and a pH of 6.25. The EOF here, however, was still too low resulting in excessive run times. Additional work had to be carried out to further optimize the electroosmotic flow.

Since the MAPS coating is known to be unstable in basic solution, it was hypothesized that the coating could be partially stripped using a moderately

basic solution to increase the number of free silanol groups and therefore increase the electroosmotic flow. A 25 mM solution of borate buffer, whose pH was raised to 11.0 with 1N sodium hydroxide, was drawn through the capillary using vacuum suction. Once again, the EOF was still too rapid.

The borate buffer treatment was repeated for another 20 minutes to give an EOF migration time of 9.5 minutes (5.8 cm/minute). Several injections were made over several days and the MAPS coating appeared to be stable. There were no significant variations in migration time or electroosmotic flow.

The reproducibility of the preparation and stripping process was studied. The MAPS-coated capillary was prepared in triplicate and the EOF determined for each capillary. Each capillary was then treated with the borate buffer for 50 minutes. The results on the EOF reproducibilty study are summarized in Table 6.1. Methanol was used as the EOF marker.

Table 6.1 Reproducibility of MAPS-coated capillaries[a]

Capillary	EOF Before Stripping (minutes)	EOF After Stripping (minutes)
1	26.5	9.5
2	28.5	11.5
3	29.5	9.75
AVG	28.2	10.25
RSD	5.42%	10.6%

[a] Conditions: 5 mM phosphate buffer, pH 6.25, 10 mM CM-β-CD, V=25 kV.

It can be concluded that the preparation of the coatings was reproducible. However, it could be necessary to fine-tune the high pH buffer treatment to get the desired EOF. With the EOF now at a suitable rate, baseline separation was obtained for desipramine and nortriptyline, as can be seen in Figure 6.5.

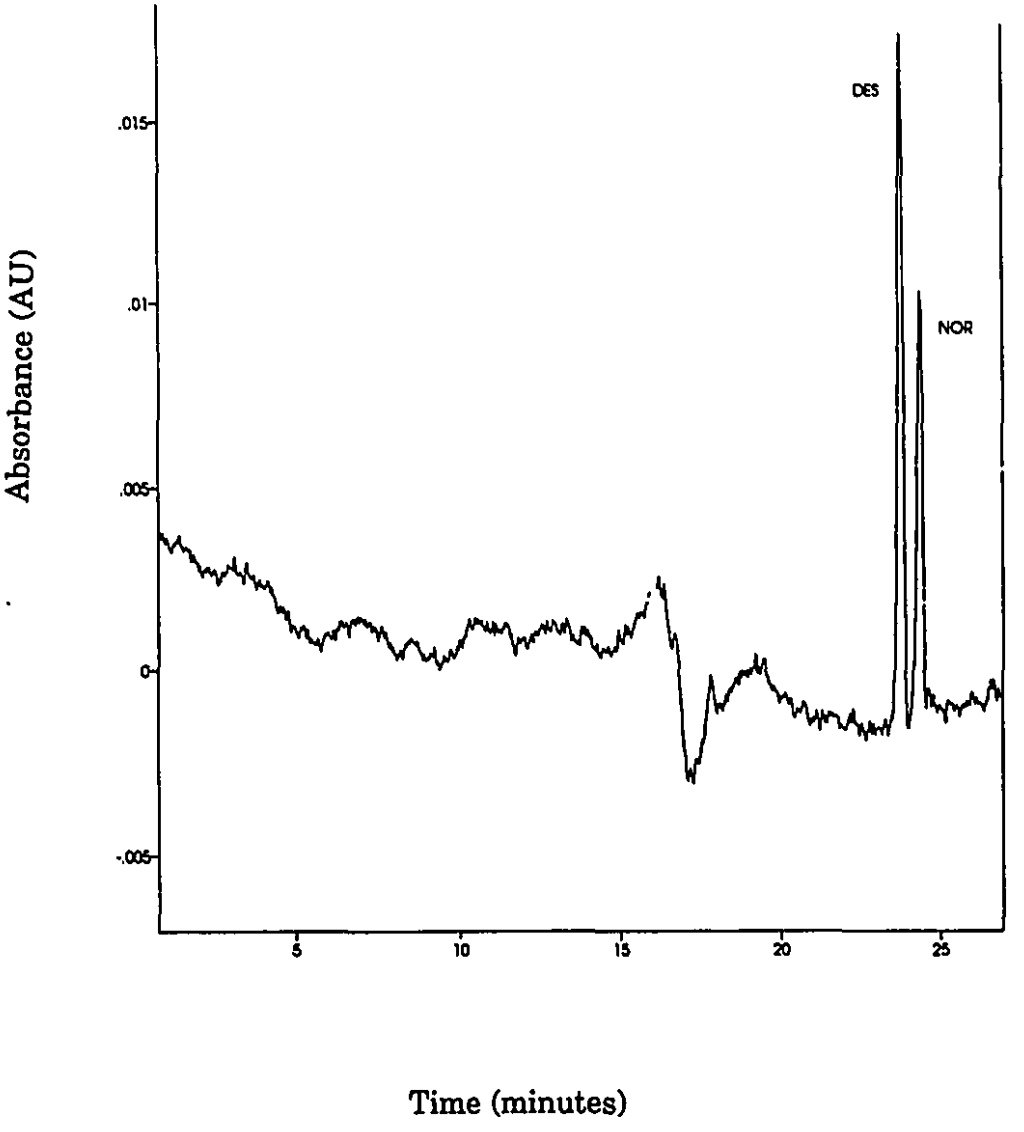

Figure 6.5 Separation of nortriptyline and desipramine. Buffer: 5 mM phosphate, 10 mM borate, 10 mM CM-β-CD, pH=6.3; V=25 kV.

Comparing this separation to Figure 6.2, it can be seen that reducing the EOF can have a significant effect on resolution. This system also successfully separated another pair of antidepressants, imipramine and clomipramine. Their separation can be seen in Figure 6.6.

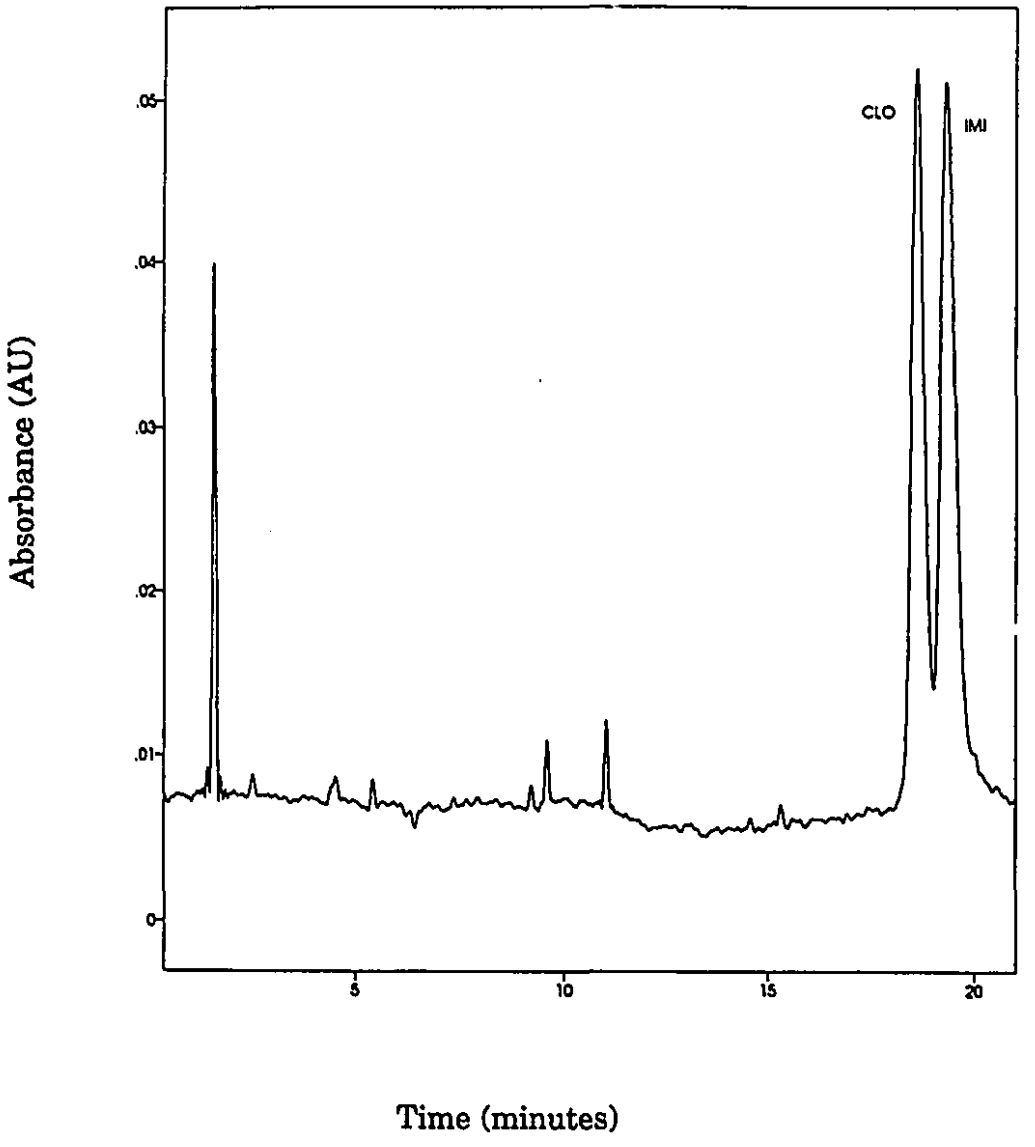

Figure 6.6 Separation of imipramine and clomipramine. Buffer: 5 mM phosphate, 10 mM borate, 10 mM CM-β-CD, pH=6.3; V=25 kV.

These separations were encouraging enough to continue the separation of the antidepressants with carboxymethyl-β-cyclodextrin using the partially-stripped MAPS-coated capillaries.

6.3.2 Separation of a Mixture of Five Antidepressants

The MAPS-coated capillary was then investigated to see if it was suitable for the separation of all nine tricyclic antidepressants. Unfortunately, it was not possible to separate all nine compounds as the cyclodextrin was not able to distinguish between all of the antidepressants. The separation of trimipramine, imipramine, and desipramine was not successful. After inclusion complexation, the differences in their molecular structure was too far removed from the cyclodextrin rim groups to have any effect on separation. As well, amitriptyline and nortriptyline migrated at the same rate as desipramine. It appears that cyclodextrin cannot distinguish between the C-N and C=C linkages on the central ring and alkyl side chain. As will be shown later, all nine compounds will be resolved, but not with carboxymethyl-β-cyclodextrin as the only complexing agent.

6.3.2.1 Structural Considerations

Carboxymethyl-β-cyclodextrin was successful in separating five of the nine antidepressants, and this separation will be discussed further. Figure 6.7 shows the separation of carbamazepine, clomipramine, desipramine, opipramol, and protriptyline.

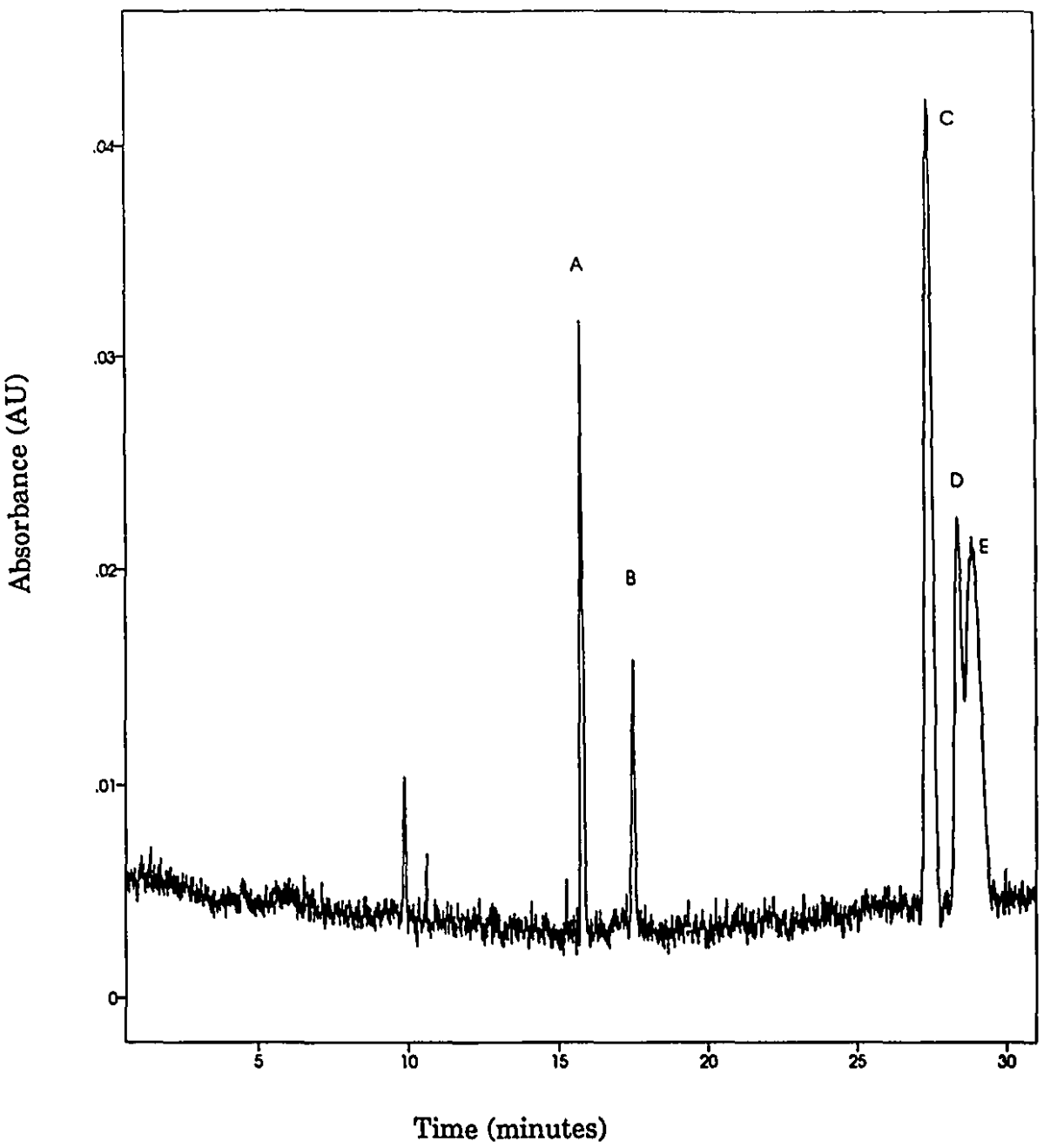

Figure 6.7 Separation of (A) carbamazepine, (B) clomipramine, (C) desipramine, (D) opipramol, and (E) protriptyline. Conditions: 10 mM phosphate, 10 mM CM-β-CD, pH=6.30; V=22 kV.

Carboxymethyl-β-cyclodextrin provided particularly good separation of carbamazepine, clomipramine, and desipramine. The amide group of carbamazepine interacts differently with the cyclodextrin rim groups than the alkyl groups of clomipramine and desipramine, resulting in a weaker inclusion complex and shorter migration times. Also, the presence of the chlorine atom at the side ring of clomipramine will influence inclusion complex strength as complexation probably occurs at this segment of the molecule. This allows for the carboxymethyl-β-cyclodextrin to distinguish it from desipramine.

The separation of desipramine from opipramol and protriptyline was probably due to the presence of the double bond in the central ring of the latter two compounds. The double bond will cause the molecules to be more rigid than the desipramine, indicating that the geometry of the tricyclic antidepressants plays an important role in inclusion complex formation. The separation of opipramol from protriptyline was not as good as the other compounds. Surmising from their structures, the variation in inclusion complex strength is probably due to the differences in the effect that the C-C and the C-N linkages have on the geometry of the molecules. Note that cyclodextrin was able to barely distinguish between the C-C and C-N linkages, but not between the C-N and C=C linkages. These structural differences are probably the basis for separation as their differences in alkyl side chain structure are likely too far removed to have any interaction with the cyclodextrin rim functional groups.

6.3.2.2 Effect of Carboxymethyl-β-Cyclodextrin Concentration

The effect of varying the carboxymethyl-β-cyclodextrin concentration on electrophoretic velocity and mobility, as well as on separation, were studied. The electropherograms in Figure 6.8 shows the effect of cyclodextrin concentration on the separation of carbamazepine, clomipramine, desipramine, opipramol,

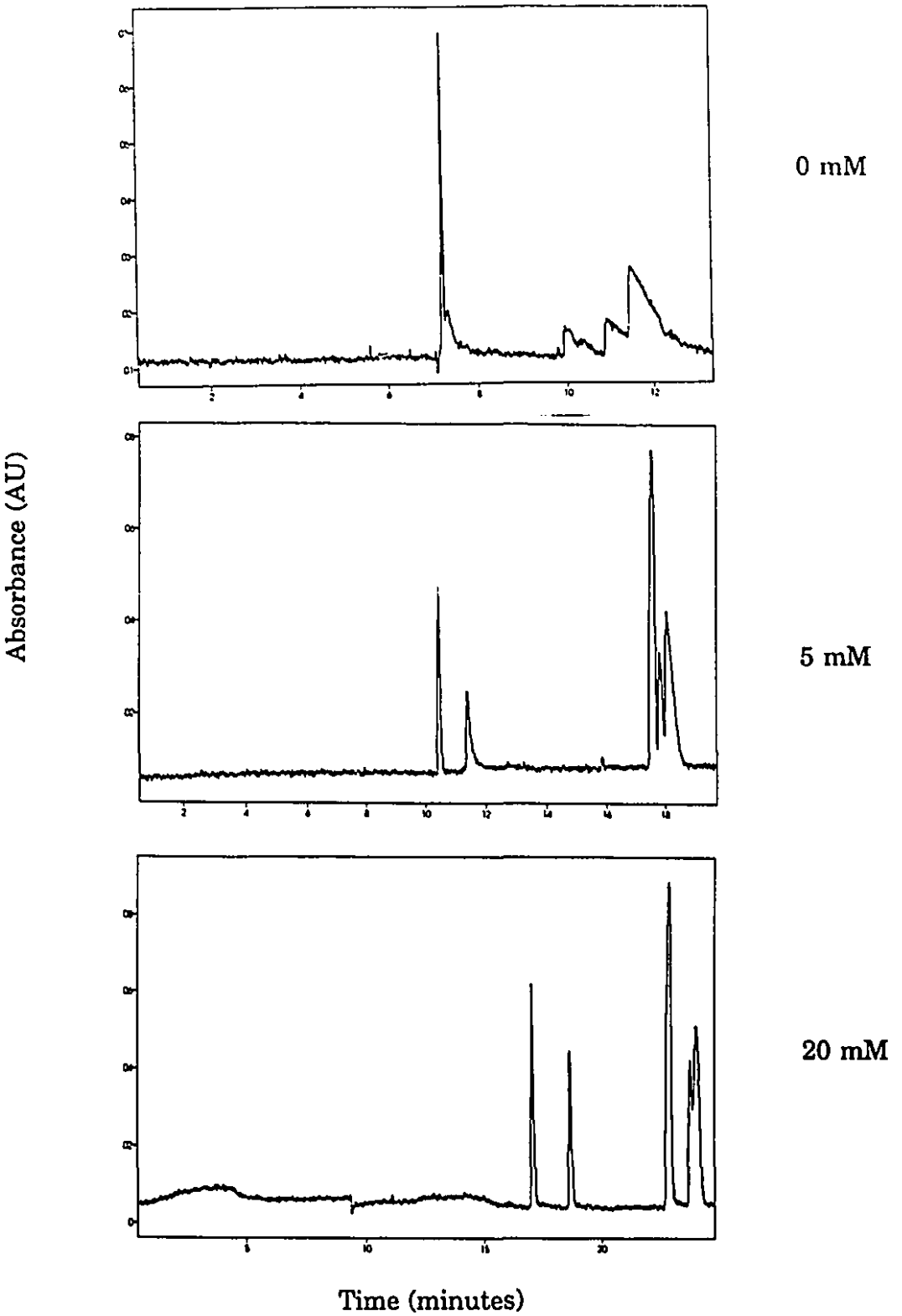

Figure 6.8 Separation of 5 tricyclic antidepressants at various CM-β-CD concentrations. Conditions: 10 mM citrate, pH=6.1, plus the indicated amount of CM-β-CD. Applied voltage was 22 kV.

and protriptyline. With no cyclodextrin in the buffer, the peaks eluted as three broad, closely-eluting peaks with excessive tailing. There was no real improvement with 1 mM CM-β-CD in the buffer; however increasing the concentration to 5 mM greatly improved the peak shapes. There was baseline resolution of carbamazepine and clomipramine while the desipramine, opipramol, and protriptyline peaks were distinguishable yet not baseline separated. At 10 mM carboxymethyl-β-cyclodextrin (see Figure 6.7), the desipramine became baseline-separated from opipramol and protriptyline; however there was not much improvement in the separation of the latter two antidepressants. Baseline separation of these two compounds was not achieved even at a cyclodextrin concentration of 20 mM.

Figure 6.9 shows the effect of cyclodextrin concentration on resolution of the various antidepressants. Four separations are featured: carbamazepine from clomipramine, clomipramine from desipramine, desipramine from opipramol, and opipramol from protriptyline.

The resolution, R_s, was calculated according to equation 6.4:

$$R_s = \frac{1}{4} \frac{\Delta\mu \sqrt{N}}{\mu_{ep} + \mu_{eo}} \qquad (6.4)$$

where $\Delta\mu$ is the difference in electrophoretic mobilities, μ_{ep} is the average mobility of the two solutes, μ_{eo} is the mobility of the electroosmotic flow, and N is the number of theoretical plates, which is given by:

$$N = 5.54 \left(\frac{t_{obs}}{t_w}\right)^2 \qquad (6.5)$$

t_{obs} is the migration time of the solute and t_w is its peak width at half height. The number of theoretical plates calculated for the partially coated 70 cm capillary was determined to be 253000, or a height equivalent of a theoretical plate (HETP) of 27.6 μm per plate.

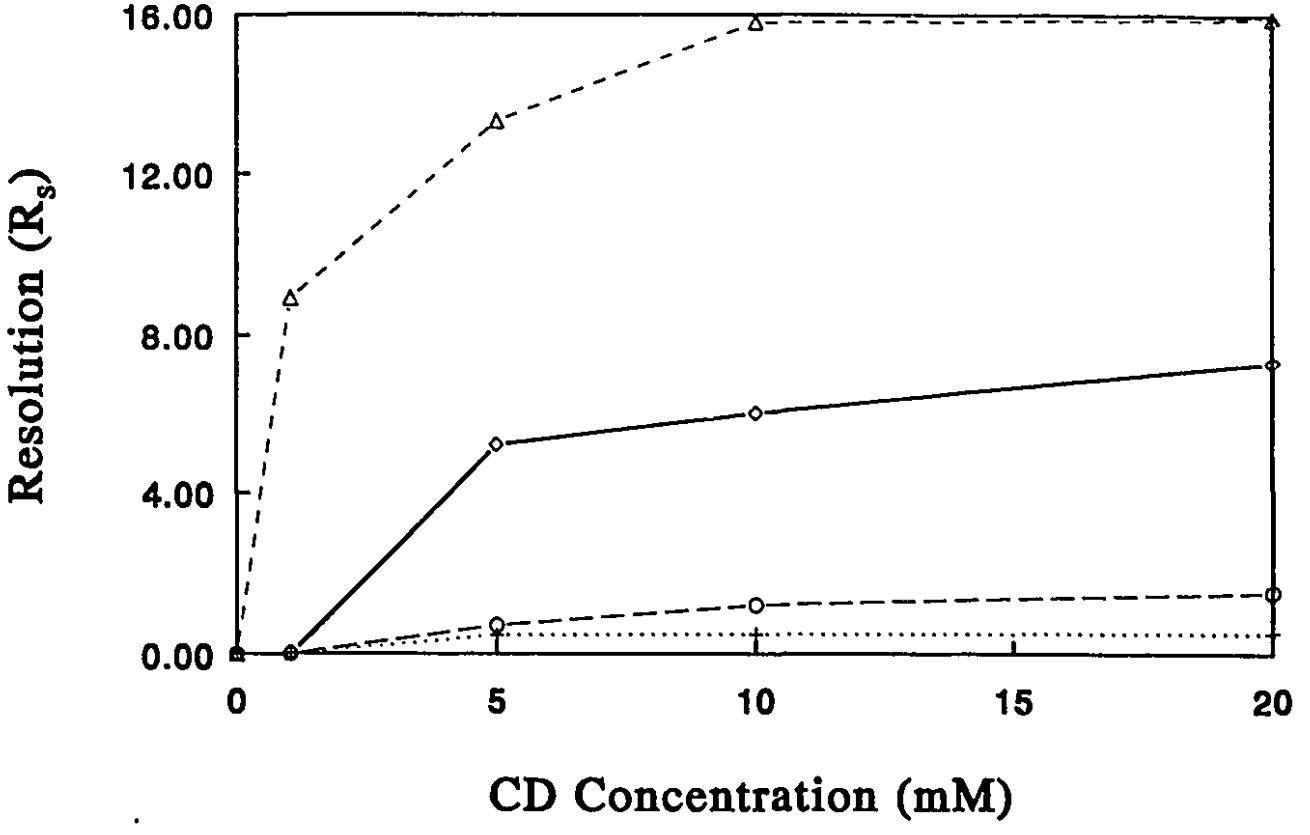

Figure 6.9 Effect of CM-β-CD concentration on resolution factor between antidepressants. Conditions same as Figure 6.8. ◊ = CAR-CLO; Δ = CLO-DES; o = DES-OPI; + = OPI-PRO.

Varying the cyclodextrin concentration will affect the electrophoretic mobility and velocity of the solutes. Figure 6.10 shows the effect of carboxy-methyl-β-cyclodextrin concentration on the electrophoretic mobility of the tricyclic antidepressants.

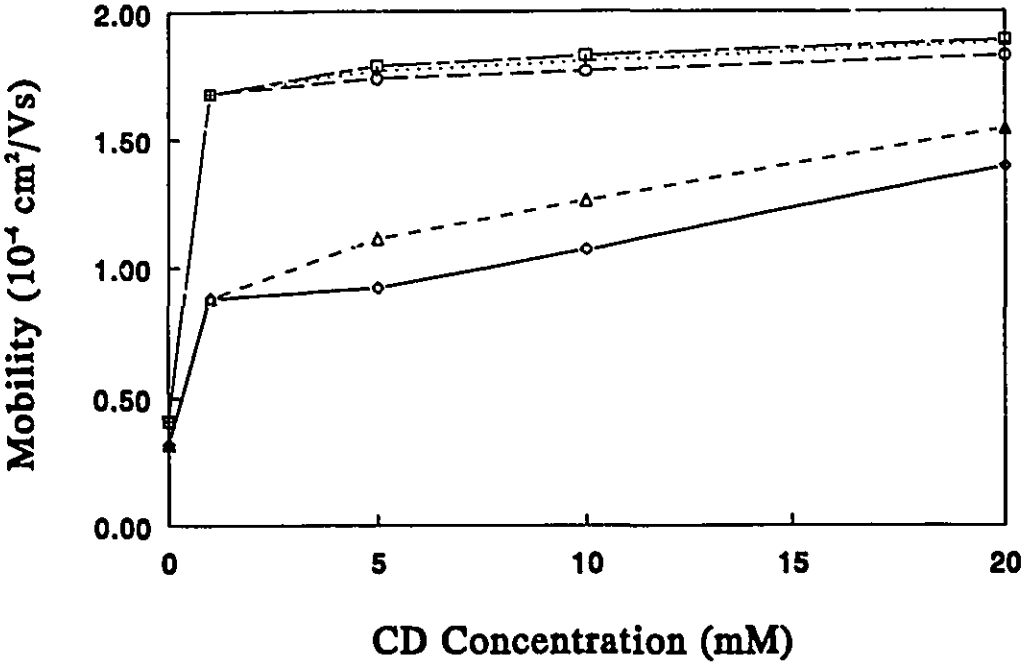

Figure 6.10 Effect of CM-β-CD concentration on electrophoretic mobility. Conditions same as Figure 6.8. ◊ = CAR; Δ = CLO; o = DES; + = OPI; □ = PRO.

With no cyclodextrin in the buffer, the antidepressants migrated towards the detector with help from the electroosmotic flow. However, upon addition of cyclodextrin to the buffer, electrophoretic mobility increased as the antidepressants complexed with the cyclodextrin. The migration time of the solutes increased significantly as the cyclodextrin migrated in the opposite direction to the EOF, thus retarding the migration of the solutes towards the detector. After the large initial increase, the mobility of all five antidepressants increased steadily with cyclodextrin concentration over the concentration range of 0 to 20 mM. This is an indication that inclusion complexes were formed with carboxymethyl-β-cyclodextrin.

6.3.2.3 Effect of pH

The effect of varying the pH on electrophoretic mobility and separation was studied. pH will have a large effect on the migration and separation of the tricyclic antidepressants. The degree of ionization of carboxymethyl-β-cyclodextrin and the electroosmotic flow are both pH dependent and will have significant influence on migration and separation.

Figure 6.11 displays the effect of pH on the migration time of the antidepressants. At a low pH of 3, the carboxyl groups on the CM-β-CD are predominantly in their protonated form and therefore will not migrate electrophoretically. Also, at this pH, the electroosmotic flow is greatly reduced as the zeta potential decreases due to the decreasing negative character of the capillary wall.

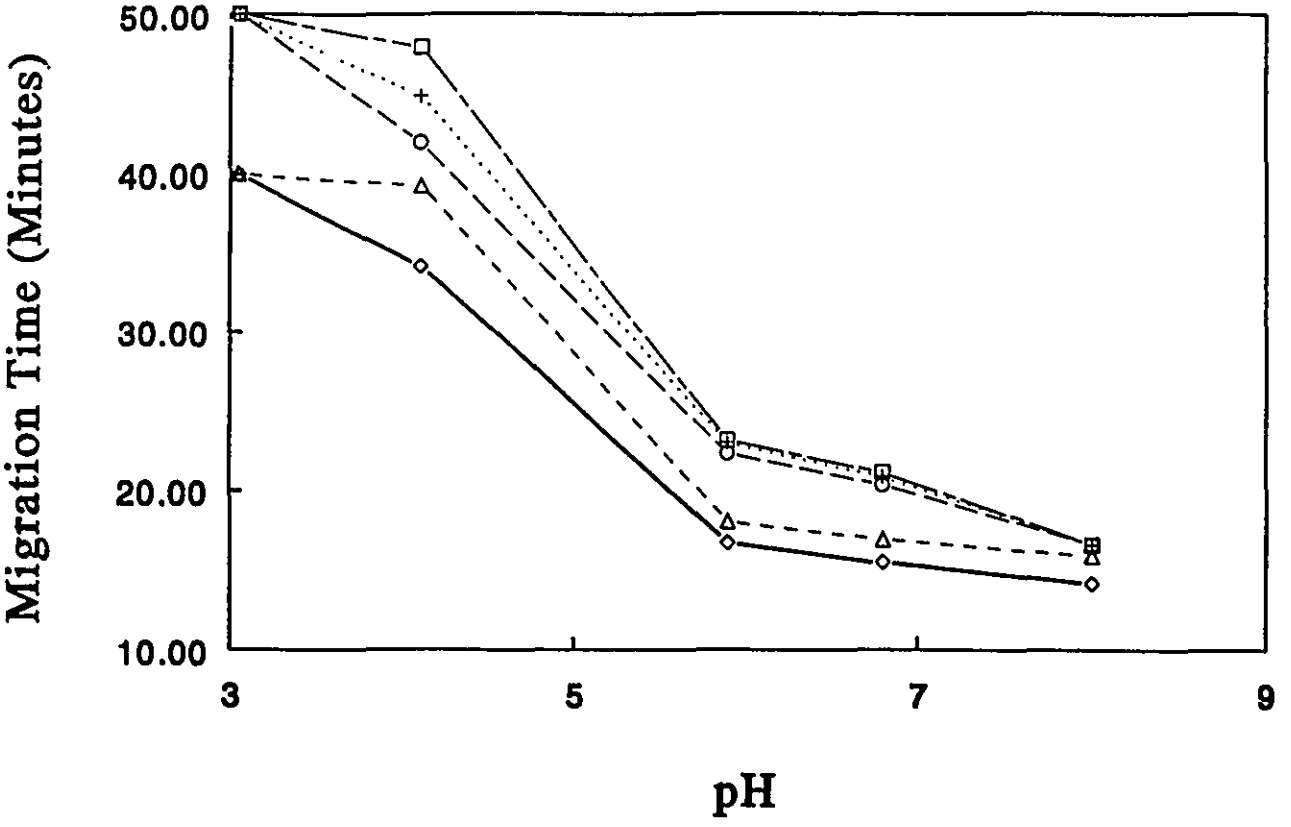

Figure 6.11 Effect of pH on migration time. Conditions: 10 mM citrate, 10 mM CM-β-CD, V=22 kV. ◊ = CAR; Δ = CLO; o = DES; + = OPI; □ = PRO.

As the pH increases, the EOF increases with the increase in zeta potential; therefore the migration time of the antidepressants decreases. This increase in electrophoretic velocity is offset somewhat by the increasing ionic character of carboxymethyl-β-cyclodextrin. The electrophoretic mobility of the cyclodextrin will increase with increasing pH; therefore the solutes will be slowed in the capillary by the cyclodextrin. The trend of decreasing migration time with increasing pH was observed until pH 8, although the effect was not as pronounced at the higher pH values. The pH was not increased above 8 as the MAPS coating is unstable above this value. At the higher pH values (above 6), increasing the pH had little effect on cyclodextrin ionization; however it did affect the electroosmotic flow.

Varying the pH also affected separation. Figure 6.12 shows the electropherograms of the separation of five antidepressants, carbamazepine, protriptyline, desipramine, clomipramine, and opipramol, at pH 4.2, 5.9, and 8.0. Note that the elution times decrease with increasing pH. At the highest pH studied here, resolution was adversely affected as the high EOF hampered complete separation. Separation was not significantly affected by pH in the range of 5-7. Below pH 5, however, band broadening arising from longitudinal diffusion due to the excessive run times resulted in wide and poorly shaped peaks. It is evident that this separation cannot be carried at the pH extremes and that a suitable pH that optimizes the degree of CM-β-CD ionization and EOF is of prime importance. The ideal pH for this separation is in the range of 5-7 where the electroosmotic flow and cyclodextrin mobility are optimized.

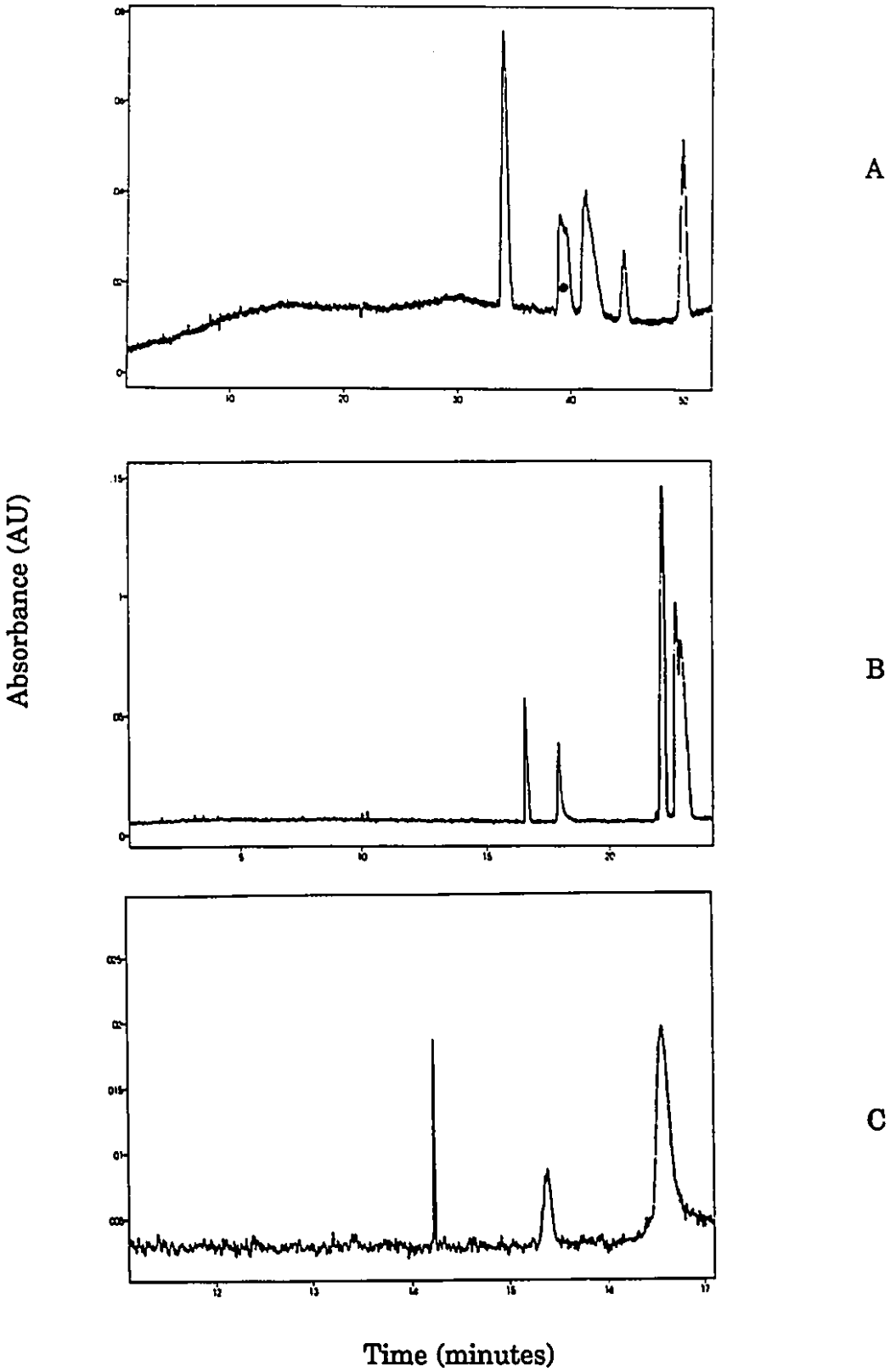

Figure 6.12 Effect of pH on separation. Conditions are same as Figure 6.11. pH of buffer was **A** 4.2, **B** 5.9, **C** 8.0.

6.3.2.4 Effect of Coating Functionality

As was mentioned before, Dougherty *et al.* [17] performed a study on the effects of wall coatings on the separation of various proteins. They investigated a C_8, a C_{18}, and a non-identified "polar" phase. The preparation of the phases was described as "proprietary" and therefore details were not provided. They concluded that these coatings reduced, but not eliminated, the electroosmotic flow. Two of these phases, C_8 and C_{18}, were compared here with coatings having propylmethacrylate and isobutyl functionalities. The separation and migration of five tricyclic antidepressants were compared for each of the coatings. The antidepressants were those investigated in the previous section, namely carbamazepine, protriptyline, desipramine, clomipramine, and opipramol. Immobilization of these coatings to the capillary wall was described in section 6.3.1 and followed the reaction in equation 6.3, where the "R" was one of the above-mentioned functional groups. The chemical structures of these groups are shown in Figure 6.13.

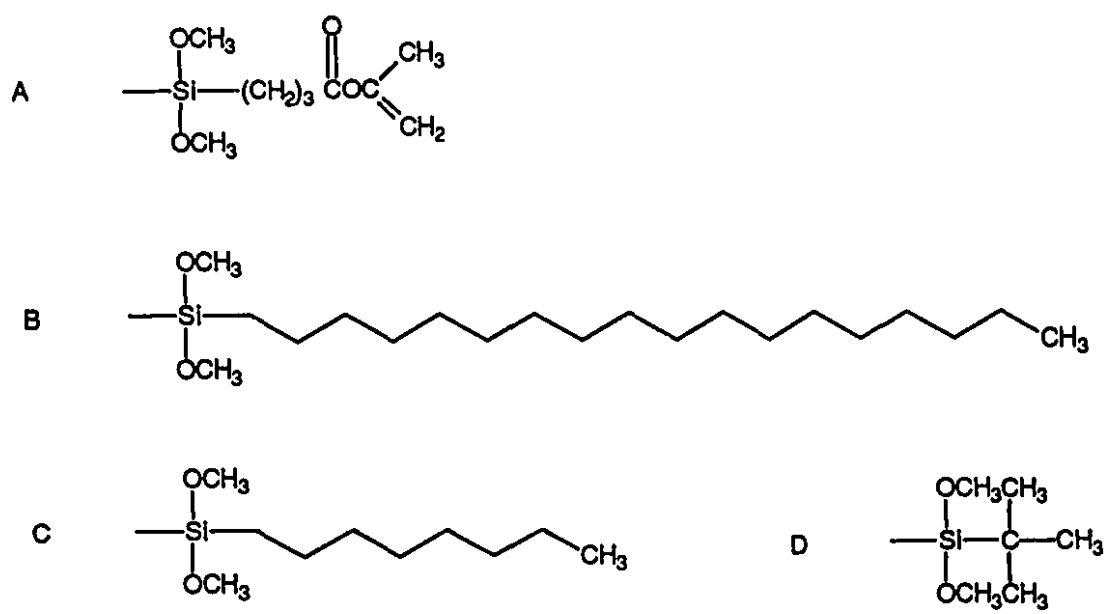

Figure 6.13 Coating functional groups. (A) propylmethacrylate; (B) C_{18}; (C) C_8; (D) isobutyl.

Each coating was successful in reducing the electroosmotic flow to some degree. Table 6.2 shows the reduction in EOF for each of the coatings. The EOF marker was methanol.

Table 6.2 Comparison of reduction of electroosmotic flow by various capillaries coatings[a]

Coating	Electroosmotic Flow (cm/minute)	% Reduction
Bare siiica	0.147	-
Isobutyl	0.095	35.4%
C_8	0.084	42.8%
C_{18}	0.071	51.7%
Propyl-Methacrylate	0.081	44.9%

[a] Conditions: 10 mM citrate buffer, pH 6.10, 10 mM CM-β-CD, V=22 kV.

The C_8 and MAPS coatings gave similar reductions in the electroosmotic flow. The isobutyl coating did not reduce the EOF as well as the other three coatings. The long alkyl chains on the other three coatings formed a network that effectively covered a good part of the capillary wall resulting in a significant decrease in EOF. The isobutyl's lack of a network due to its much shorter alkyl chain did not cover the capillary wall enough to reduce the EOF to the same degree as the other three. The longer alkyl chains of C_{18} had a greater effect of reducing the EOF than the other three coatings. The isobutyl, C_8, and propylmethacrylate coatings gave comparable resolution and migration times, as shown in Figure 6.14.

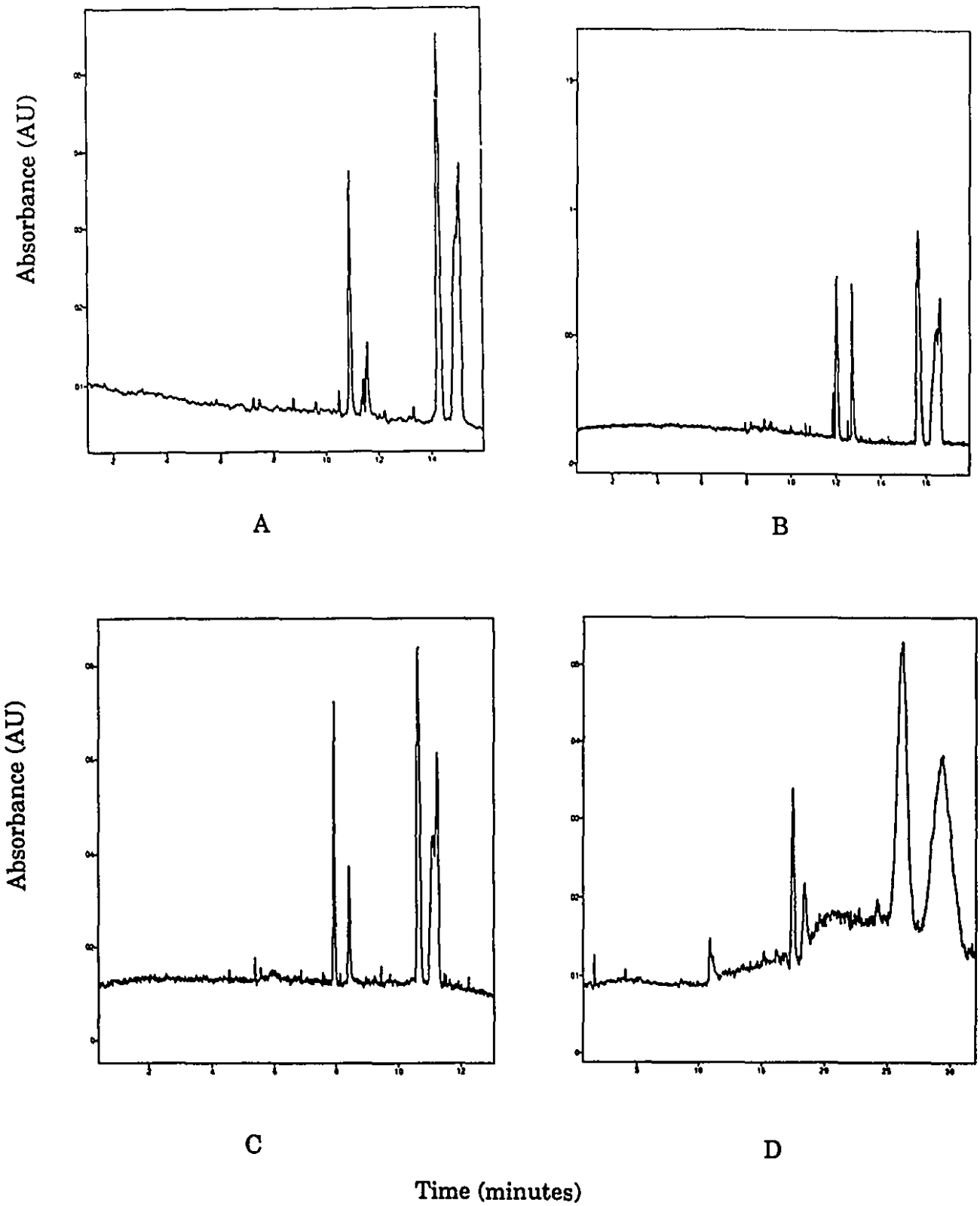

Figure 6.14 Effect of coating on separation. Conditions same as Figure 6.7. (A) isobutyl; (B) C_{18}; (C) C_8; (D) propylmethacrylate

It was observed that the solutes had exhibited increased migration time on the C_{18}-coated capillary. This was probably due to hydrophobic interactions between the antidepressants and the long alkyl chains of C_{18} on the capillary wall. This effect was not as pronounced with the C_8 coating. This contrasts with the findings of Dougherty and co-workers [17] who reported that C_8- and C_{18}- coatings had the same effect on the separation of proteins. The differences could be due to the nature of the analytes under consideration or to the coating technique which they did not describe. Ideally, the capillary surface should be very hydrophillic to minimize hydrophobic interactions and retention as mass transfer will cause band broadening [1]. However, hydrophobic coatings are preferred for reducing the EOF so the effect the coatings had on the electro-osmotic flow was of greater importance than possible interactions with the solutes.

6.3.3 Separation of a Mixture of Nine Antidepressants

As was described in section 6.2.2, the use of charged cyclodextrin as the lone complexing agent was not capable of distinguishing between all nine antidepressants under investigation. The functional groups on the non-resolvable compounds did not interact distinctly with the cyclodextrin rim groups, therefore carboxymethyl-β-cyclodextrin alone is not suitable for their separation. Another additive would be necessary for complete separation. This reagent must be able to differentiate between the small differences in the alkyl side chains away from the tricyclic ring system. An obvious choice would be the use of a micellar system as the combination of inclusion complexation with the cyclodextrin and hydrophobic interactions within the micelles might be able to distinguish between all nine compounds. While the original aim of this work was to perform the separations without having to use micelles in order to avoid

the band broadening problems associated with MEKC, it was clearly not possible for CM-β-CD to distinguish between all antidepressants.

Sodium dodecyl sulfate (SDS) was investigated as a buffer additive. The combination of SDS and CM-β-CD was successful in separating all nine tricyclic antidepressants. The separation mechanism is now a complex one with the solute partitioning between the cyclodextrin and the micelle. One would expect that the solutes would prefer the relatively more hydrophobic micellar structure over the cyclodextrin cavity. However, inclusion complexation does play a significant role in separation as SDS alone cannot separate all the compounds whereas a mixture of cyclodextrin and SDS does successfully separate all nine of them. The separation of carbamazepine, trimipramine, opipramol, clomipramine, imipramine, desipramine, amitriptyline, nortriptyline, and protriptyline can be seen in Figure 6.15.

The capillary coating used in this separation was not the MAPS-coated one used in the separations of five antidepressants described in section 6.3.2. Initially, the micelle-cyclodextrin system was attempted with a bare-silica capillary which resulted in seven of the nine antidepressants being resolved. A MAPS-coated capillary was then examined to reduce the electroosmotic flow; nevertheless this resulted in only a slight improvement but not in a complete resolution. The EOF was then eliminated by preparing a 4% polyacrylamide coating. The polarity of the applied voltage was reversed and the cyclodextrin-micelle system produced the separation of all nine compounds.

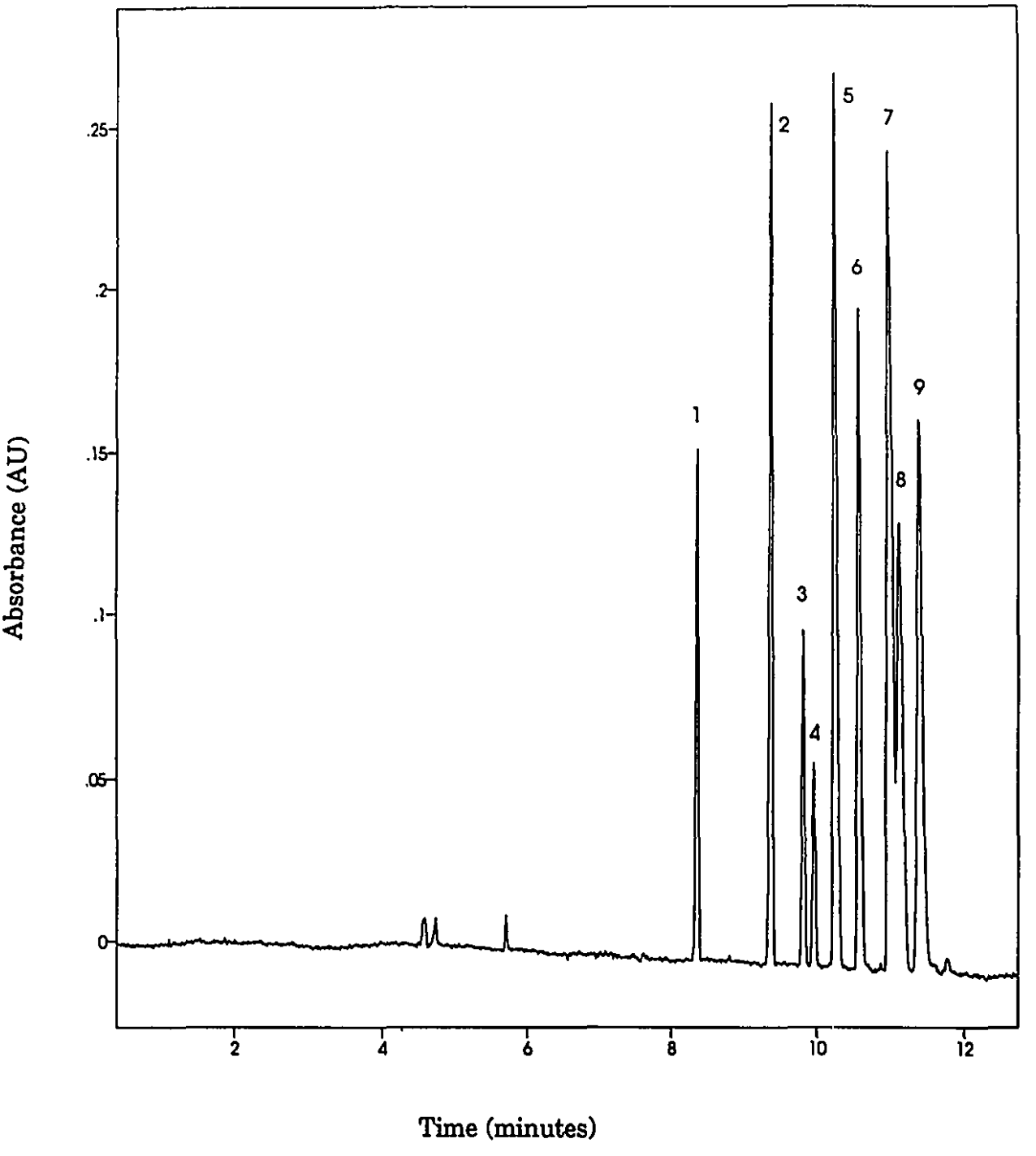

Figure 6.15 Separation of nine tricyclic antidepressants. Buffer was composed of 10 mM citrate, 20 mM SDS, 10 mM CM-β-CD, pH=6.5; V = -20 kV.
1 Protriptyline; **2** Trimipramine; **3** Imipramine; **4** Desipramine; **5** Opipramol; **6** Carbamazepine; **7** Amitriptyline; **8** Nortriptyline; **9** Clomipramine.

Note that the elution order of the five antidepressants from section 6.3.2 has changed as a result of the presence of the SDS micelles. This is an indication that the micelle is playing a significant role in separation. To ensure that SDS was not acting alone in the separation, a buffer with no cyclodextrin was prepared and this resulted in three peaks for nine components. With cyclodextrin alone in the buffer (no SDS), there were three peaks for nine components. Complete separation occurs only when a mix of the two is utilized as the antidepressants partition between the micelle and cyclodextrin. The complexing equilibria for the solutes are shown in equations 6.6 and 6.7, where AD represents the antidepressant, CD the cyclodextrin, and SDS the micelle:

$$AD + CD \overset{K_i}{\rightleftharpoons} AD\text{-}CD \qquad (6.6)$$

$$AD + SDS \overset{K_m}{\rightleftharpoons} AD\text{-}SDS \qquad (6.7)$$

It is not expected that the complexes would interact with the other complexing reagent, as in equations 6.8 and 6.9:

$$AD\text{-}SDS + CD \rightleftharpoons AD\text{-}SDS\text{-}CD \qquad (6.8)$$

$$AD\text{-}CD + SDS \rightleftharpoons AD\text{-}CD\text{-}SDS \qquad (6.9)$$

In the first case, the micelle-solute complex is much too large to fit into the cyclodextrin cavity. In the second scenario, there is little interaction between the micelle and the cyclodextrin-solute complex. The hydrophillic outer surface of the cyclodextrin torus will not interact with the hydrophobic

micellar structure [1]. Furthermore, the negative charge on both the SDS and the CM-β-CD will aid in their repulsion.

The effect of carboxymethyl-β-cyclodextrin concentration on separation was then studied. Figure 6.16 shows the electropherograms for the separation at three different cyclodextrin concentrations. The SDS concentration was kept constant at 20 mM. With no cyclodextrin the buffer, the antidepressants eluted as 3 peaks, evidence that the micelle was not solely responsible for separation. Increasing the SDS concentration to 50 mM with no CM-β-CD produced four peaks. Resolution began to improve immediately at a cyclodextrin concentration of 1 mM. At 5 mM CM-β-CD, resolution is almost complete. Separation at 10 mM cyclodextrin can be seen in Figure 6.15 and features the optimal separation achieved. As the cyclodextrin is increased to 15 mM, there is a loss in resolution as the peaks become slightly wider as the increase in Joule heating from the ionic cyclodextrin increases band broadening. Also note that the migration time decreases with increasing cyclodextrin concentration, evidence that the cyclodextrin does have an effect on separation. The shorter retention time at 15 mM can also be a reason for the loss in resolution.

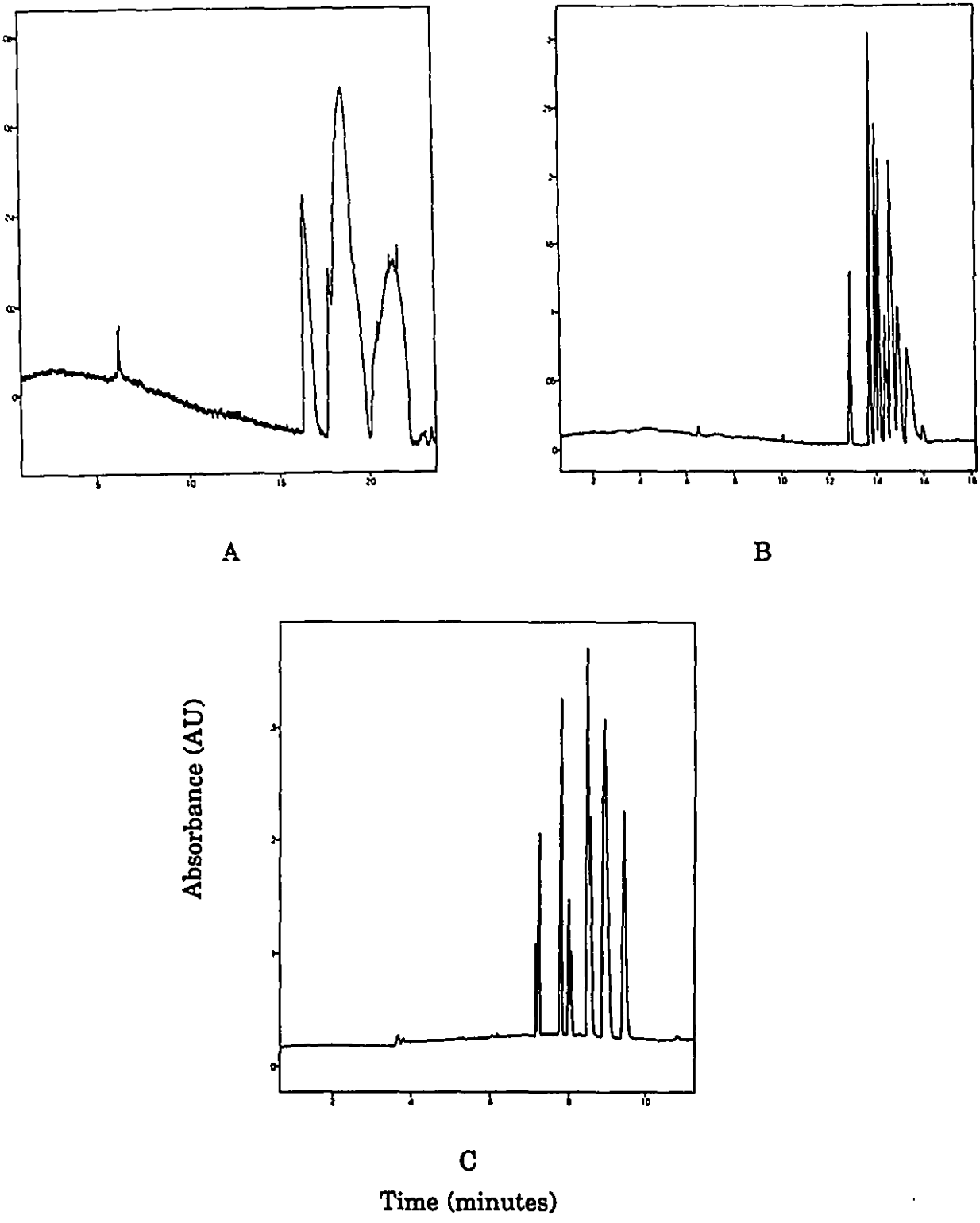

Figure 6.16 Effect of CM-β-CD concentration on separation. Conditions: 10 mM citrate, 20 mM SDS, pH=6.5, plus **A** 0 mM CM-β-CD; **B** 5 mM CM-β-CD; **C** 15 mM CM-β-CD.

Finally, the effect of pH on the separation and migration of the nine tricyclic antidepressants with carboxymethyl-β-cyclodextrin was studied. The pH was varied from 3.5 to 7.0, a range in which the degree of ionization of the antidepressants was not greatly affected. However, the degree of ionization of the carboxymethyl-β-cyclodextrin is dependant on pH and this affected both migration and separation. Figure 6.17 shows the separation of the nine antidepressants at different pH values.

At pH 3.5, the cyclodextrin is predominantly neutral therefore it has little influence on separation. The retention times are long and resolution is poor. Increasing the pH to 4.5 increases the ionization of the cyclodextrin and therefore increases the electrophoretic mobility of the inclusion complex. This results in decreased migration times and less band broadening due to diffusion. However, only seven of the antidepressants were resolved. Increasing the pH to 6.0 further reduced the migration times and resolved all nine peaks, although imipramine, desipramine, and opipramol were not baseline resolved. A further increase of pH to 7.0 did not improve separation but did reduce the migration time slightly. The pH of the buffer was not raised above 7.0 as the polyacrylamide coating was unstable above this pH.

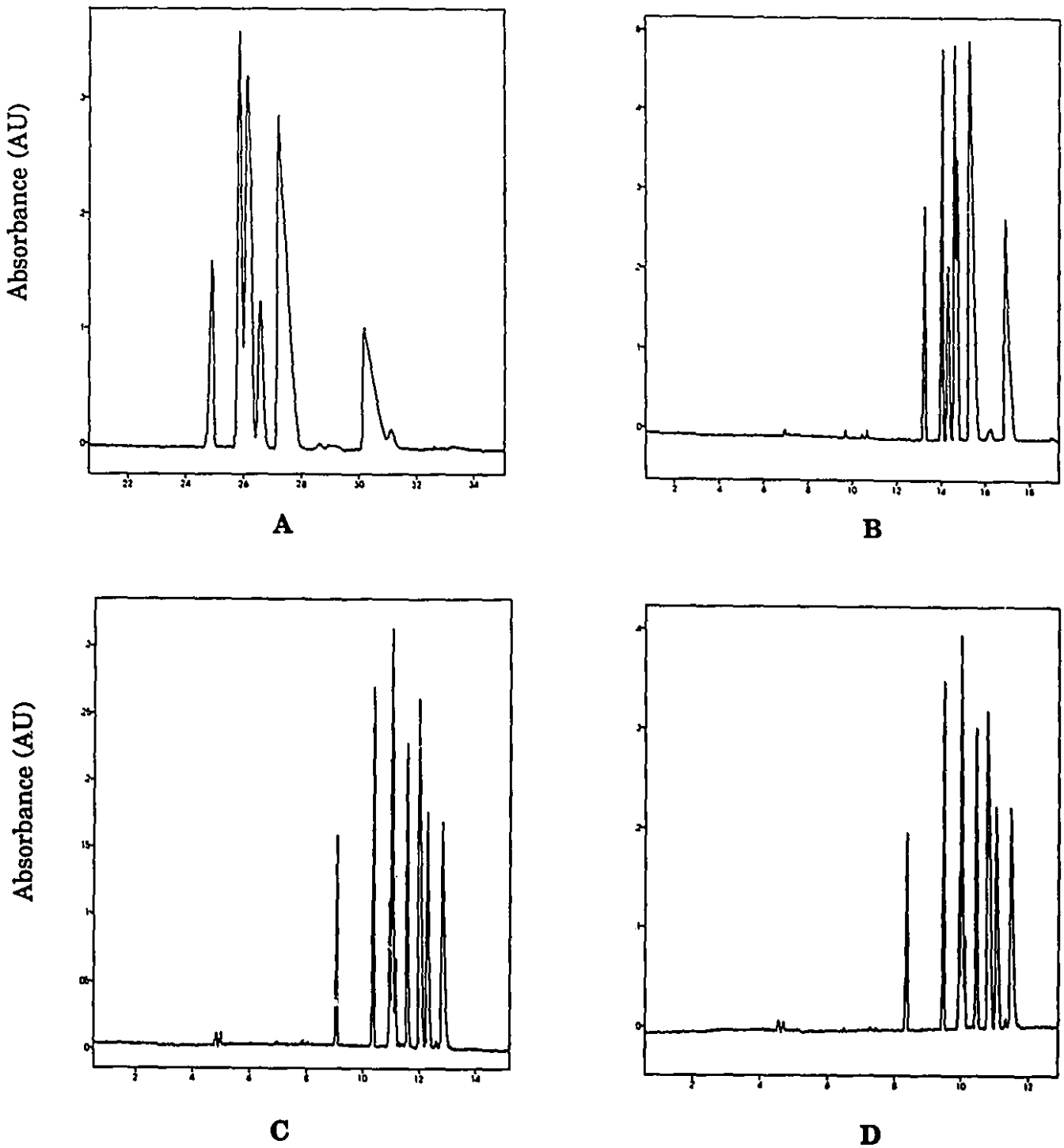

Figure 6.17 Effect of pH on separation. Conditions: 10 mM citrate, 20 mM SDS, 10 mM CM-β-CD, pH= **A** 3.5; **B** 4.5; **C** 6.0; **D** 7.0.

6.4 CONCLUSIONS

The charged cyclodextrin, carboxymethyl-β-cyclodextrin, was successful in the capillary electrophoretic separation of a series of tricyclic antidepressants. The cyclodextrin alone was unable to separate all nine compounds under investigation. It was successful in the separation of carbamazepine, protriptyline, desipramine, clomipramine, and opipramol while using a 3-(trimethoxysilyl)propyl methacrylate capillary coating to reduce the electroosmotic flow. Other suitable coatings investigated were isobutyl and octylsilane phases. An octadecylsilane coating was also investigated; however it resulted in significant band broadening and was considered unsuitable as a coating for this separation.

pH and cyclodextrin concentration were important factors to consider in this separation as the pH affected both the cyclodextrin ionization and electroosmotic flow. An ideal pH would be in the range of 6-7. Cyclodextrin concentration affected both the migration and separation of the antidepressants. The ideal cyclodextrin concentration was found to be 10 mM.

All nine antidepressants were resolved using the charged cyclodextrin in the micellar electrokinetic chromatography mode with sodium dodecyl sulfate as the micelle. Neither the cyclodextrin nor the micelle alone were successful in resolving the whole series of compounds under investigation while a combination of both was effective in their separation. In order to avoid the band broadening usually associated with MEKC, a 4% polyacrylamide capillary coating had to be used to eliminate the EOF. The solutes partitioned between the charged cyclodextrin and micelle as they migrated towards the detector. The pH of the buffer had to be maintained between 5-7. A pH below 5 adversely affected the ionization of the cyclodextrin while a pH above 7 would ruin the polyacrylamide coating.

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CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

In this work, the following contributions to original knowledge are claimed.

1. The separation of dinitrophenyl and dinitrobenzoyl amino acids enantiomers by high performance liquid chromatography with cyclodextrin as a mobile phase additive. It was shown that enantioselectivity was greatly improved when β-cyclodextrin was replaced with dimethyl-β-cyclodextrin. The effects of cyclodextrin, methanol, and buffer concentration, as well as pH, were studied.

2. β-Cyclodextrin, dimethyl-β-cyclodextrin, and hydroxyethyl-β-cyclodextrin were used as mobile phase additives in the liquid chromatographic separation of equilin from estrone and estrone from 16α-, 2-, and 4-hydroxyestrone. Effects of cyclodextrin and methanol concentration were studied. Inclusion complex strengths for estrone, 16α-, 2-, and 4-hydroxyestrone with all three cyclodextrins were determined.

3. A series of fat-soluble vitamins were separated by reversed-phase HPLC and micellar electrokinetic chromatography (MEKC) using cyclodextrins in the mobile phase and running buffer. Separations included D_2 and D_3, E and E acetate, and α-,β-,γ-, and δ-tocopherol. Cyclodextrins were found to greatly affect the chromatographic retention and electrophoretic migration times for vitamins D_2, D_3, E, E acetate, K_1, K_2, and K_3.

4. The use of charged carboxymethyl-β-cyclodextrin was used in the capillary electrophoretic separation of five similarly structured tricyclic antidepressants. The effects of cyclodextrin concentration and pH on retention and separation were studied. Both parameters were critical for the separation. A stripping process to increase the number of free silanol groups to increase the EOF was developed for a 3-(trimethoxysilyl) methacrylate coated capillary.

5. Carboxymethyl-β-cyclodextrin was used in combination with a micellar pseudophase comprising of sodium dodecyl sulphate to separate a series of nine tricyclic antidepressants by capillary electrophoresis. Effects of cyclodextrin concentration and pH were studied.

Appendix A

Publications

The work presented in this thesis has resulted in the following manuscripts:

[1]. B.J. Spencer and W.C. Purdy, "High-Performance Liquid Chromatographic Separation of Derivatized Amino Acid Enantiomers Using Modified β-Cyclodextrin as a Mobile Phase Additive." *Analytical Letters*, 28(10), 1865-1881 (1995).

[2]. B.J. Spencer and W.C. Purdy, "High-Performance Liquid Chromatographic Separation of Equilin, Estrone, and Estrone Derivatives with Cyclodextrins as Mobile Phase Additives." *Journal of Liquid Chromatography*, 18(20), 4063-4080 (1995).

[3]. B.J. Spencer and W.C. Purdy, "Comparison of the Separation of Fat-soluble Vitamins by High Performance Liquid Chromatography and Micellar Electrokinetic Chromatography." In preparation.

[4]. B.J. Spencer, W. Zhang, and W.C. Purdy. "Capillary Electrophoretic Separation of Tricyclic Antidepressants Using Anionic β-Cyclodextrin as a Buffer Additive." In preparation.