STUDIES  IN  HIGH  PERFORMANCE

LIQUID  CHROMATOGRAPHY


by


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## DECLARATION

This thesis is the original composition of the author's work, unless stated otherwise, and has not been submitted previously for any other degree.

I attended the following post-graduate lecture courses:-

"Chemistry at its most colourful"

ICI Organic Division, by:-

Dr. C.V. Stead, Mr. F. Hall, Dr. D.B. Baird,

Dr. C.W. Greenhalgh, Dr. R. Price and Dr. P. Bamfield.

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TO   MY   PARENTS

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ABSTRACT

A brief history of chromatography from its inception to the present day is presented in Chapter 1, with special emphasis being given to the development of liquid chromatography.

Chapter 2 describes the different techniques of high performance liquid chromatography. Improvement of the column efficiency with better understanding of kinetics of column chromatography is also described from a theoretical point of view in this Chapter.

Chapter 3 shows that resolution in chromatography depends not only upon the kinetics but that thermodynamics also plays an important role. The basic equations of the thermodynamics of different techniques used in HPLC are shown in this Chapter.

Thermodynamics of Reversed Phase Chromatography (RPC) and development of different techniques of Ion Pair Chromatography (IPC) in general, and of RP-IP HPLC in particular is discussed in Chapter 4.

The first steps toward development of some novel forms of RP-IP-HPLC referred to as "Dynamically Generated Hydrophobic Stationary Phase" using cationic surfactant for separation of anionic species and aromatic hydrocarbons on acidic surfaces is described in Chapter 5. The thermodynamics of separation is also briefly explained in this Chapter.

Separation of cationic species (such as amines) as well as aromatic hydrocarbons were achieved on a basic surface using anionic surfactants. These experiments are the subject of Chapter 6. The effect of two different carbon chain length surfactants on retention behaviour are also investigated.

Chapter 7 shows the use of non-ionic surfactants for the first time in liquid chromatography. These surfactants proved to be applicable to both acidic and basic surfaces. Excellent separations of aromatic hydrocarbons and a ketone on silica and alumina surfaces with particular attention on the effect of different surfactant carbon chain length are shown in this Chapter.

In Chapter 8, we have shown a possible mode of separation of cationic species on acidic surfaces by the use of double surfactants (anionic-non ionic). It is also shown that anionic species can equally be separated on acidic surfaces when double surfactants were applied. The results of this Chapter were compared with those of Chapter 5.

General conclusions on the usefulness of these novel techniques and their advantages and disadvantages over chemically bonded stationary phases, and some future aspects of "Solvent Generated Hydrophobic Stationary Phase" technique are also discussed in the last part of this thesis.

# CONTENTS

# CHAPTER 1

## Literature Survey of Chromatography

1.1     The history of Chromatography may be said to have its origin with paper chromatography when Pliny used papyrus impregnated with an extract of gall nuts for the detection of ferrous sulfate (1). Then column chromatography was discovered and named in 1906 by Michael Tswett (2), a Russian botanist, when he separated a solution of coloured leaf pigments by passing it through a column packed with adsorbent chalk particles. The individual pigments passed down the column at different rates and were separated from each other. The separated pigments were easily distinguished as coloured bands. The technique was not followed up until the 1930's when Kuhn and Lederer (3) and Reichstein and Van Euw (4) again used adsorption chromatography for the separation of natural products.

Alongwith the development of column chromatography, Martin and co-workers (5) extended the work of Neuberger (6), who attempted in 1938 to separate the neutral amino acids by chromatography and observed that the partition coefficient of acetylated amino acids between water and an immiscible organic solvent differed for the various amino acids. Martin and Synge (7) used water as a stationary liquid phase spread over the surface of the adsorbent silica gel and immiscible with the mobile phase, chloroform. The sample components partitioned themselves between the two liquid phases according to their solubilities. In the same paper, they developed their well known "Theoretical Plate" theory of column efficiency.

They also pointed out that improved performance would be achieved in liquid chromatography by the use of smaller particles and higher pressures, and that the liquid mobile phase could be replaced by a gas. For this and related studies on paper chromatography, Martin and Synge received the Nobel Prize in Chemistry in 1952.

Thin Layer Chromatography (TLC) was first referred to in 1931 by two Russian workers, Ismailov and Schraiber (8). Little notice was taken of the method until ten years later when two American chemists described the separation of terpenes in essential oils by TLC.

Thin Layer Chromatography, as it is presently known, began to attract attention through the work of Kirchner and his associates, starting in 1951 (9). The procedure was not generally accepted in its early years because available media and apparatus for coating the plates lacked uniformity. It was not until 1958, when Stahl (10, 11) described equipment and efficient sorbents for the preparation of plates, that the effectiveness of the technique for separation was shown.

However, the importance of applications of column chromatography to separation of mixtures of colourless and complex homogeneous substances meant that design of equipment for chromatography was inevitable. This equipment was required to have a wide range of applications, speed of analysis, operational simplicity and, finally sensitivity of detection (in the range of nano-grams). For these requirements, Martin and James (12) developed and introduced the technique referred to as gas chromatography, and it rapidly became widely used for separation of compounds which could be volatilised.

GC was the first "high performance" chromatography mode, and advantage of GC arose from the fact that the low viscosity of the carrier gas, which permits rapid diffusion of the vapourized solute in the column and the nearly instantaneous attainment of equilibrium between the mobile and stationary phase. The low viscosity of the carrier gas also permits large flow rates which result in fast elution of the solutes from the column. Then the development of sensitive detectors able to respond accurately to high performance separation systems as well as efficient data recorders to record detector response were necessary. Thus high performance gas chromatographs soon became sophisticated instruments. Application and greater understanding of the theory of gas chromatography was due to the work of Giddings in 1965 (13).

Liquid chromatography remained relatively neglected during this period, although Hamilton, etal (14) who worked on amino acid ion exchange chromatography and applied the known theory of GC to LC. They showed that as particle diameter was reduced peaks became sharper and narrower as expected from the Van Deemter equation (15). Later Snyder substantially advanced the understanding of the thermodynamic basis of adsorption chromatography (16).

However it was only subsequent to this that the theory and technology of GC made its full impact in LC. Thus in the early 1960's Giddings (13) showed that "the theoretical framework for GC which involves the inter-relation of particle diameter, pressure drop, mobile phase velocity and column efficiency, could be applied equally well to liquid chromatography if the correct scaling factors were used.

Firstly, because liquids are a hundred times as viscous as gases, operating pressures would have to be increased by this factor going from GC to LC. Secondly, because of the $10^3$ to $10^4$ times smaller rates of diffusion of substances in liquids compared to gases the size of the particles of support materials have to be reduced in proportion to the square root of this ratio in order to give equivalent rates of mass transfer. Finally, because of the much smaller geometrical size of the optimal LC column, special attention would have to be paid to design of injector and detector improvements."

Then between 1967-1969, Huber (17) and Horvath, Preiss and Lipsky (18) described the first high performance liquid chromatographs. Operating at high pressure (up to 5000 psi) these instruments overcame the effect of higher liquid viscosities relative to gas viscosities and gave analysis times comparable with GC. Development of sensitive ultra-violet photometric detectors was initiated at this time, and these allowed detection of nano-gram quantities of absorbing compounds, but were insensitive to compounds with little or no U.V. absorbance. Also at this time the refractive index detector (first used for gel permeation chromatography) was improved and redeveloped for HPLC. Subsequently spectrofluorimetric (19) and electrochemical (20,21) detectors have become common, and HPLC/mass spectrometric interfaces are now being developed (22,23).

However, without improvement in column materials, modern HPLC would not be possible. Significantly improved performance was achieved by Kirkland (24) who developed a "controlled surface porosity packing."

He argued that band broadening due to the slow mass-transfer within the stagnant mobile phase held in the pores of the packing could be minimised by two approaches. Firstly, superficially porous particles (also porous layer or pellicular beads) could be used which have solid cores and a thin porous outer shell. These materials minimise the depths of pores within the particle so that the solute molecules can rapidly move into and out of the shallow pool of stagnant mobile phase in the porous layer. These materials were marketed under the trade-name of Zipax, but suffered from low surface area and therefore low sample capacity. One of the several ways of reducing the effect of stagnant mobile phase is to reduce the particle size. Both theory and practice have shown that $\simeq 5\mu m$ represents a good compromise between efficiency, pressure drop, analysis time, and reproducibility of packing (25). However, early work indicated that it was increasingly difficult to pack particles when the size was reduced below about $40\mu m$. For the small particles ($<13-20\mu m$) dry packing techniques were impossible and slurry packing (26,27) techniques were developed and more recently upward slurry packing was introduced by Bristow (28) who also reviewed the methods in general use.

The development of microparticles in place of pellicular materials (29) greatly improved the speed of liquid chromatography. Subsequently the development of chemically bonded stationary phases in which the nature of the adsorbent was modified by bonding organic groups to the adsorbent surface extended the range of application to HPLC. The first bonded phases for HPLC had in fact been prepared by Halasz and Sebestian who reacted silica with alcohols (30).

Subsequently, materials with greater hydrolytic stability were prepared by bonding organosilanes to the surface of silica and a wide range of such phases is now commercially available. These chemically modified adsorbents can be used for "reversed phase" and "ion exchange" chromatography. The preparation and applications of chemically bonded stationary phases in HPLC have been discussed in several review articles (31-34).

Compared with classical column chromatography, where columns were gravity fed and separations could take hours or even days, HPLC offers analysis times of 5-30 minutes, comparable with GC. HPLC is particularly suited to the analysis of those compounds which are not readily handled by GC. For example, highly polar compounds, ionized compounds and thermally labile compounds can be analyzed at ambient temperatures by HPLC without prior derivatization. Polymeric samples can also be analyzed with specially designed microparticulate wide pore silica gels or other related rigid materials. Sample clean-up is usually much less of a problem with HPLC than GC and biological fluids can often be directly injected onto an HPLC column. The necessity for sample pretreatment is also much reduced since aqueous solvents can be used in HPLC. A combination of these factors has meant that in the decade since its inception, HPLC has already made an enormous impact in the pharmaceutical chemistry and is becoming increasingly important in environmental and clinical analysis. It can certainly be regarded as completely complemental to GC analytical procedures.

Recently a novel form of HPLC has been developed in which use

is made of the ability of organic ions to form ion pairs which can be extracted from an aqueous phase into an organic phase. The technique is known as ion pair chromatography. In one form of the technique small amounts of the salts of long chain organic amines or quaternary ammonium compounds are added to the eluent. This salt is adsorbed by hydrocarbon chains bonded to silica to form what amounts to a dynamic ion exchanger (35, 36). The technique is often called "Soap Chromatography" and may be seen as a special case of a group of LC techniques in which the mode of separation is largely determined by the strong adsorption of a trace component added to the eluent which provides what may be termed a "dynamically coated stationary phase."

This thesis deals mainly with novel aspects of this technique in which non-ionic materials rather than ionic materials are used as these additions to the eluent. For this purpose investigations have been made in the adsorption of anionic, cationic, and non-ionic surfactants on the surfaces of various supports, including silicas, aluminas and zirconias and into the chromatographic properties of such modified supports. These new chromatographic systems have been applied to the development of novel separations of high efficiency and selectivity for hydrocarbons, amino acids, short peptides, and other biologically important compounds.

## CHAPTER   2

### 2.   Definitions and Kinetics of Liquid Chromatography

#### 2.1      Definitions

The aim of column technology in HPLC may be defined as the achievement of the optimum combination of resolution of solutes, speed of elution, and economic use of pressure.  The design of all other components of an HPL Chromatograph must be governed by the most desirable configuration and operating conditions for the column.

The key to resolution in any form of chromatography is the proper combination of the differential migration of solutes and the control of band spreading.  Fortunately, control of the migration rates, and of band spreading, can be treated almost independently, since the former is governed by thermodynamic (or equilibrium) considerations, while the latter is governed by kinetic considerations.

Before proceeding with the detailed arguments, the main parameters used to specify chromatographic systems will be first defined and then explained.

#### 2.2      Retention and Column Capacity Ratio

After a small volume of sample solution has been injected at the column inlet, the following solvent phase moves the sample through the column packing.  The individual components undergo sorption and desorption on the packing, thereby slowing their motion in varying amounts depending on their affinity for the packing.  Each component X is distributed between the stationary phase(s) and the mobile phase (m) as it passes down the column.

FIGURE 2-1

Chromatogram of a three-component mixture at constant eluent flow rate.
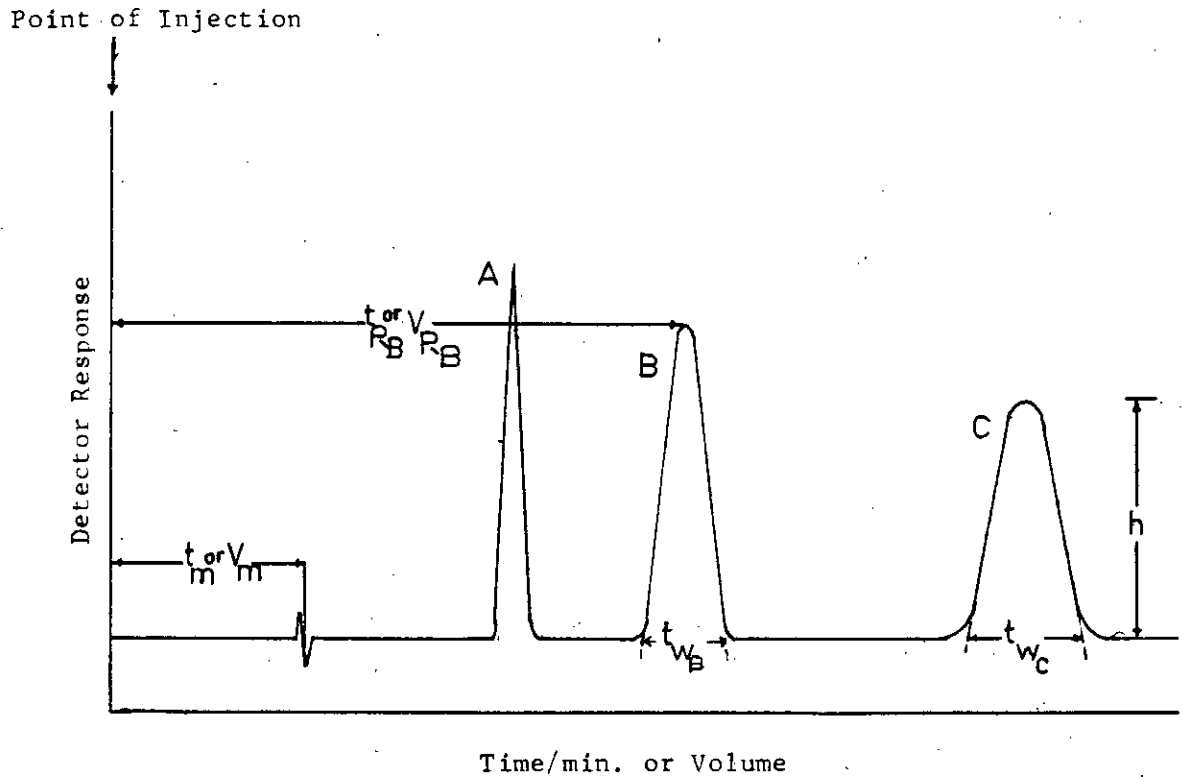


$t_m$ = time for solvent to traverse the column.

$t_{RB}$ = retention time of substance B

$t_{WB}$ = peak base width of substance B

h = peak height

Units can also be given in terms of volume rather than time, $V_m$, $V_{RB}$, $V_{WB}$, and so on.

According to:

$$X_m \; \rightleftharpoons \; X_s \qquad\qquad 2\text{-}1$$

The corresponding distribution coefficient for the component X is given by:

$$K_x \;=\; \frac{(X)_s}{(X)_m} \qquad\qquad 2\text{-}2$$

Where $(X)_s$ and $(X)_m$ indicate the molar concentration of X in stationary and mobile phases respectively.

A large value of $K_x$ indicates that the component favours the stationary phase and moves slowly through the column, whereas for small values of $K_x$ the component favours the mobile phase and moves quickly through the column. If one measures the concentration of each component as it exits from the column and plots it as a function of the volume of mobile phase passed through the column, a chromatogram results. A chromatogram for a hypothetical three-component sample is depicted in Figure 2-1.

Now let the average velocity of sample band X be $U_x$ it should be clear that $U_x$ depends upon the fraction R of molecules X in the mobile phase, that is:

$$U_x \;=\; U.R \qquad\qquad 2\text{-}3$$

Where U is the solvent velocity in the column.

If the fraction of molecules X in the mobile phase is zero, no migration can occur, and $U_x$ is zero. If the fraction of molecules X in the mobile phase is one (i.e. all molecules X are in the mobile phase), then molecules X move through the column at the same rate as solvent molecules, and $U_x = U$.

Therefore from the above discussion and equation 2-3, we can write:

$$U_x = U \left(\frac{N_m}{N_m + N_s}\right) = U \left(\frac{1}{1 + \frac{N_s}{N_m}}\right) = \frac{U}{1 + k'} \qquad 2\text{-}4$$

Where $N_s$ and $N_m$ the mole fraction of solute X in stationary and mobile phases respectively and

$$k' = \frac{N_s}{N_m} = \frac{V_s}{V_m} \cdot \frac{(X)_s}{(X)_m} = \varphi \, K_x \qquad 2\text{-}5$$

$$\varphi = \frac{V_s}{V_m} = \text{Phase ratio}$$

and $K_x$ is the equilibrium distribution ratio of X between mobile and stationary phases which is explained in 2-1.

The quantity $U_x$ can be related to retention time of solute, $t_r$ and column length, L, by the relationship:

$$t_r = \frac{L}{U_x} \qquad 2\text{-}7$$

and

$$t_m = \frac{L}{U} \qquad 2\text{-}8$$

Where $t_m$ is the time for an average solvent molecule (or other unretained compounds) to move from one end of the column to the other. Hence equations 2-3, 2-4, 2-7 and 2-8 give:

$$R = \frac{t_m}{t_r} = \frac{1}{k' + 1} \qquad 2\text{-}9$$

or

$$k' = \frac{t_r - t_m}{t_m} \qquad 2\text{-}10$$

The capacity ratio from equation 2-10 is a more practical quantity than R, since it can be determined directly from the elution chromatogram and schematically is shown in Figure 2-1.

FIGURE 2-2

Major dispersion mechanisms in chromatography.
Indicates two molecules initially adjacent,
"S" represents their separation after operation
of the dispersion process.



(a)

(b)

(c)
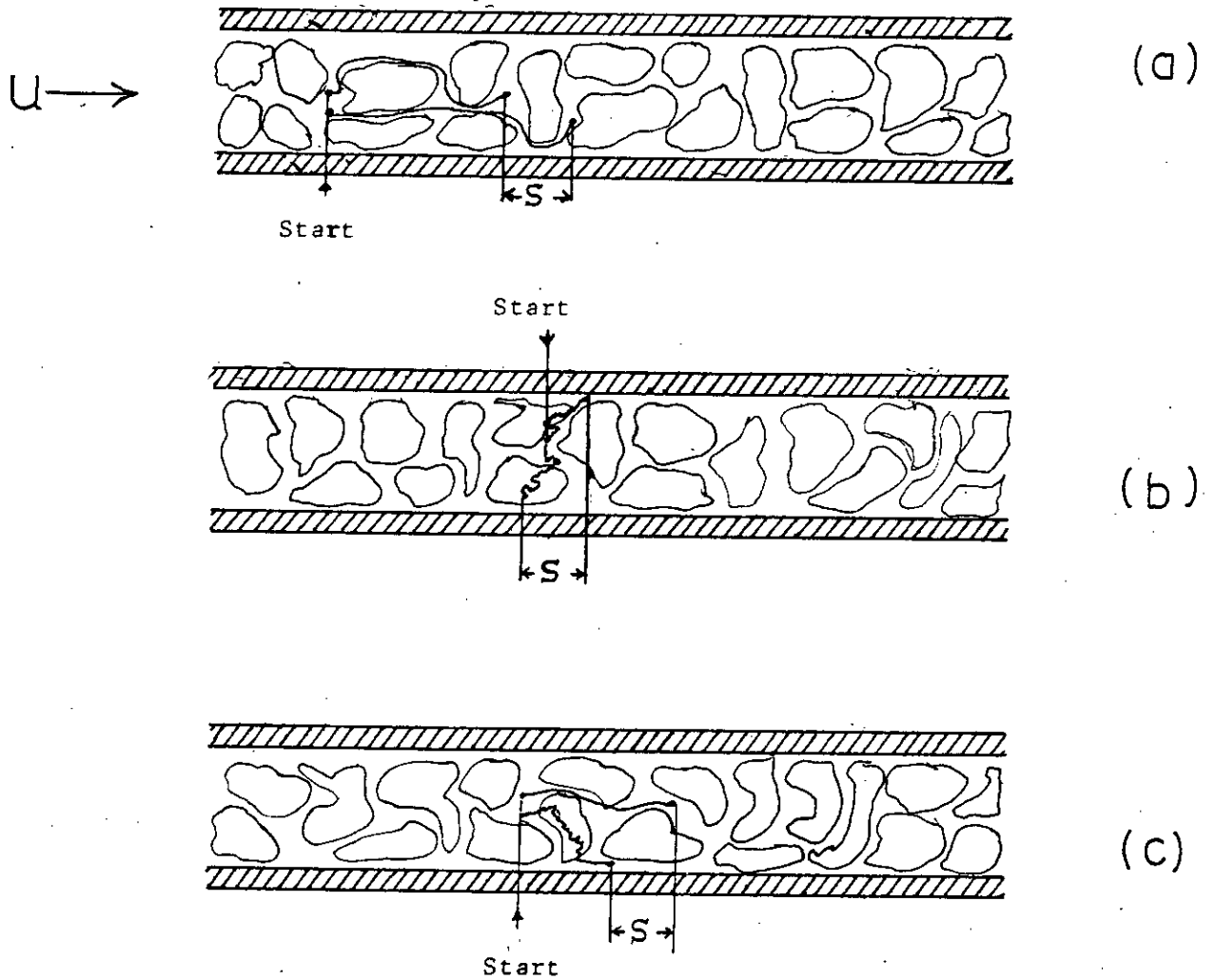
(a) Dispersion by tortuous flow in mobile phase.

(b) Dispersion by axial diffusion (static conditions shown).

(c) Dispersion by slow equilibration between mobile and
stationary phases.

k' can be defined in terms of retention volume, $V_r$ where:

$$V_r = F_c \cdot t_r \qquad \text{2-11}$$

Where $F_c$ is the flow rate of the mobile phase.

Thus

$$k' = \frac{V_r - V_m/F_c}{V_m/F_c} = \frac{V_r - V_m}{V_m} \qquad \text{2-12}$$

Where $V_m$ is the elution volume of an unretained peak.

Therefore from equation 2-12 we have:

$$V_r = V_m (1 + k') \qquad \text{2-13}$$

Finally by replacing k' from equation 2-13 by equation 2-5:

$$V_r = V_m + K_x V_s \qquad \text{2-14}$$

Equation 2-14 is the fundamental equation for any chromatographic process.

## 2.3 Number of Theoretical Plates and Plate Height

Column efficiency is related to the rate of band broadening as the solute travels through the column. As illustrated in Figure 2-1, all molecules do not move at the same speed. Dispersion of molecules generally results in a Gaussian profile for the concentration of a separated zone. The rate of travel of the centre of profile or elution band represents the average rate of travel of a solute molecule. Small deviations from the mean value are brought about by the finite rate of solute mass-transfer between the mobile and stationary phases, the different flow paths through the stationary phase caused by irregular packing in the bed, and axial (longitudinal) diffusion in the direction of flow. These effects are illustrated in Figure 2-2.

The plate height (H) is defined as the increase of zone variance, $\sigma$, with the distance migrated. This is schematically represented in Figure 2-3.

or

$$\sigma \propto \sqrt{Z} \qquad \text{2-15}$$

FIGURE 2-3

Migration of sample band. (a) chromatographic
response Vis time of migration. (b) chromatographic
response against distance migrated.



(a)

(b)
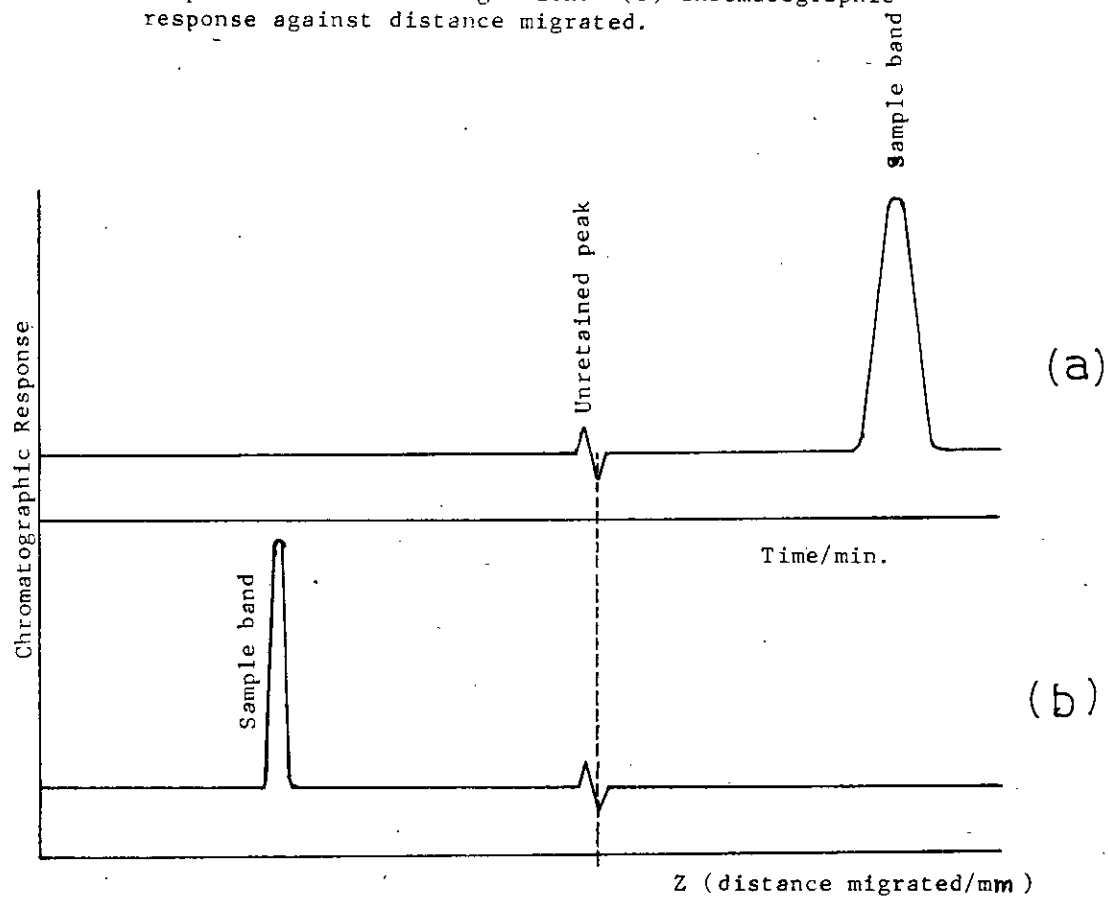
So from Figure 2-3, it can be written:

$$\sigma_Z^2 = HZ \qquad\qquad 2\text{-}16$$

Where H is a constant and called the height equivalent to a theoretical plate.

Therefore at the end of the column we have:

$$\sigma_L^2 = HL \qquad\qquad 2\text{-}17$$

Number of plates is given by:

$$N = \frac{L}{H} = \left(\frac{L}{\sigma_L}\right)^2 \qquad\qquad 2\text{-}18$$

In equation 2-13, $V_m$ can be defined as:

$$V_m = L.A. \qquad\qquad 2\text{-}19$$

Where A is the cross-sectional area of the column. Therefore by

substituting equation 2-19 in 2-13 we have:

$$L = \frac{V_r}{A\,(1 + k')} \qquad\qquad 2\text{-}20$$

and also

$$\sigma_L = \frac{\sigma_v}{A\,(1 + k')} \qquad\qquad 2\text{-}21$$

Equation 2-22 is defined as the combination of equations 2-18, 2-20

and 2-21, so:

$$N = \left(\frac{V_r}{\sigma_v}\right)^2 = \frac{t_r^2}{\sigma_t^2} \qquad\qquad 2\text{-}22$$

The base width of the elution band ($W_t$) is equal to $4\sigma$ (standard

deviation), and by replacing $W_t$ with $\sigma$ , it is possible to achieve

the fundamental column efficiency (plate number) equation:

$$N = 16\left(\frac{t_r}{W_t}\right)^2 = 5.54\left(\frac{t_r}{W_{t\frac{1}{2}}}\right)^2 \qquad\qquad 2\text{-}23$$

Where $W_{t\frac{1}{2}}$ is the peak width at half height assuming a Gaussian profile of the zone.

## 2.4    Resolution in Chromatography

The resolution $R_s$ of two adjacent bands 1 and 2 is equal to the distance between the two band centres, divided by average band width:

$$R_s = \frac{t_{r2} - t_{r1}}{\frac{1}{2}(W_1 + W_2)} \qquad\qquad 2\text{-}24$$

The quantities $t_{r1}$ and $t_{r2}$ refer to the $t_r$ values of solutes $X_1$ and $X_2$ and $W_1$ and $W_2$ to their $W_t$ values. Larger values of $R_s$ infer better separation. For adequate separation of Gaussian peaks of roughly equal size, $R_s$ must have a value greater than about 1.5. Relative separation of two adjacent bands $t_{r2} - t_{r1}/\overline{t_r}$ arises from the capability of a chromatographic system to distinguish between two components, and it is a thermodynamic quantity governed by the relative solute distribution between the mobile and the stationary phases. But the relative band width $(W_t/\overline{t_r})$ is related to the efficiency of the chromatographic

process. Unlike separation, efficiency is a kinetic parameter and can be increased by better column design as well as by the other factors which will be discussed later.

By substituting for W in terms of N in equation 2-24, a general resolution equation may be derived which relates $R_s$ to $\alpha$ ($\alpha = \frac{k_2'}{k_1'}$ the column selectivity), k', and N, given by:

$$R_s = \underset{\text{(a)}}{\frac{1}{4}} \cdot \underset{\text{(a)}}{\frac{\alpha - 1}{\alpha}} \cdot \underset{\text{(b)}}{\frac{k_2'}{k_2' + 1}} \cdot \underset{\text{(c)}}{\sqrt{N}} \qquad\qquad 2\text{-}25$$

Equation 2-25 shows that resolution is a function of three separate factors: (a) the selectivity factor, (b) a retention factor, and

(c) an efficiency factor. These factors (a), (b) and (c) can be adjusted independently. The first two factors are purely thermodynamic, while the number of theoretical plates is controlled only by kinetic considerations. It is evident from equation 2-25 that $R_s$ approaches zero (resolution is lost), as N or k' approach zero or as $\alpha$ approaches one. Any increase in $\alpha$, N, or k' favours better resolution.

## 2.5    Band Broadening Contribution to H

The first equation relating plate height to fluid velocity was given by Van Deemter, et al (15) for gas chromatography. Van Deemter's equation suggested that there are three independent mechanisms which can be related to peak dispersion, which are briefly explained here:

### (i) Eddy Diffusion

The Eddy Diffusion term arises from irregular flow through the packed particles in a column. The solute proceeds through the channels between the particles by many interconnected paths that differ in their tortuosity and degree of constriction. Because of the possible paths, solute molecules arrive at the column exit at different times. Therefore it was proposed that the plate height related to Eddy diffusion could be given as:

$$H_{ed} = 2\lambda dp \qquad\qquad 2\text{-}26$$

Where $\lambda$ is a packing characterisation factor.

Plate height due to eddy diffusion was no longer independent of flow in the introduction of reduced parameters by Giddings (38) and Knox (39). This and the non-equilibrium theory will be briefly explained later.

(ii) <u>Longitudinal Molecular Diffusion in Mobile Phase</u>

Longitudinal molecular diffusion in mobile phase gives a contribution to H shown below:

$$H_D = \frac{2\ Dm\ \gamma_m}{U}$$

Where $D_m$ is the solute diffusion coefficient in the mobile phase, $\gamma_m$ is the tortuosity factor, and U is the fluid velocity ( $U = \frac{L}{t_o}$ )

(iii) <u>Solute Mass Transfer</u>

Solute mass transfer may be in the stationary phase $(H_{sp})$ or in the "stagnant" mobile phase contained in the pores of the column $(H_{sm})$ (16,40), and these may both be sources of band broadening. The mass transfer rate can be increased by (a), decreasing the mean diffusion path through which the solute must pass (that is, decreasing the pore depth or the particle size), (b) increasing the rate of solute diffusion by decreasing the viscosity of the media through which it passes, (c) decreasing the thickness of the stationary phase so that the molecule can diffuse into and out of it very rapidly, or (d) lowering the k' value of the molecule so that it spends less time in the stationary phase. The general form of the plate height contribution arising from this phenomenon was given by Van Deemter as:

$$H_{sp} = q_s \cdot \frac{k'.ds^2.\ U}{(1 + k')2 \cdot D_s} \qquad 2\text{-}27$$

$$H_{sm} = q_{sm} \cdot \frac{dp^2.\ U}{D_m}$$

Where $d_s$ is the thickness of the film of stationary phase and $D_s$ is the diffusion coefficient of solute in the stationary phase and $q_{sm}$

is the coefficient of the solute in longitudinal mobile phase held
with the pores of the particle.

Since $H_{tot}$ is the summation of all the above distinct plate
height contributions, it may be given as:

$$H_{tot} = H_{ed} + H_D + H_{sp} + H_{sm} \qquad 2\text{-}28$$

Equation 2-28 can be simplified to:

$$H_{tot} = A + B/_U + CU \qquad 2\text{-}29$$

Equation 2-29 was introduced by Keulemans and Kwantes (41).
A, B and C are independent parameters and can be adjusted to get the
minimum plate height and velocity as below:

$$H_{min} = A + 2 \,.\, (B.C)^{\frac{1}{2}} \qquad 2\text{-}30$$

and

$$U_{min} = (\frac{B}{C})^{\frac{1}{2}} \qquad 2\text{-}31$$

## 2.6    Random Walk Theory

The random walk model was developed by Giddings and is discussed
at length in his book, "Dynamics of Chromatography" (13). This model
uses the laws of statistics to treat the random movements of solute
molecules within a chromatographic column.

He explained that if a number of molecules (objects) start to
move from exactly the same starting location, then after a series of
random movements the concentration of these objects will develop a
Gaussian distribution profile. Giddings modified the Van Deemter
equation to fit the random walk theory as below:

$$H = 2 \frac{(\gamma_m.D_m + \gamma_s.D_s.k')}{U} + \sum \frac{1}{\frac{1}{2} . \lambda .dp + \frac{D_m}{\omega_i.dp2.U}} +$$

$$\quad\quad\quad (a) \quad\quad\quad\quad\quad\quad (b)$$

$$\frac{q.U.d^2p.k'}{(1 + k')2 .D_s} \quad\quad\quad 2\text{-}32$$

$$(c)$$

Where $\gamma_m$ is termed the obstructive factor for molecular diffusion and $\gamma_s$ is a diffusion parameter when $D_s$, diffusion in the stationary phase cannot take place in a direct unobstructed path. $\omega_i$ is a velocity constant and Giddings has suggested numerical values for $\omega_i$. q is introduced to take account of the precise shape of the stationary phase pool and for a uniform film, q has the value of $2/3$.

Equation 2-32 can be simplified as:

$$H = \frac{B}{U} + \sum_i \frac{1}{\frac{1}{a} + \frac{1}{b.U}} + C.U \quad\quad\quad 2\text{-}33.$$

$$\quad\quad (a) \quad\quad\quad\quad (b) \quad\quad\quad\quad (c)$$

Where the three factors a, b, and c are modified longitudinal diffusion, coupling of diffusion and Eddy diffusion in the mobile phase, and mass transfer in the stationary phase from the Van Deemter equation respectively.

2.7    Non-Equilibrium Theory

The development of the generalised non-equilibrium theory described by Giddings (13) led to a more thorough understanding of the factors contributing to band-spreading in chromatography and hence to design of better chromatographic columns.

In the non-equilibrium theory of chromatography the movement of the solute through the column is treated as a random walk, that is,

FIGURE   2-4

Illustration of the influence of local non-equilibrium on
band dispersion.   Dashed lines: Actual concentration profile.
Solid lines: Equilibrium concentration profile.

(Reproduced by permission of Ref.13)

Stationary Phase

Interface

Mobile Phase

———————   Equilibrium concentration
– – – – – –   Actual concentration
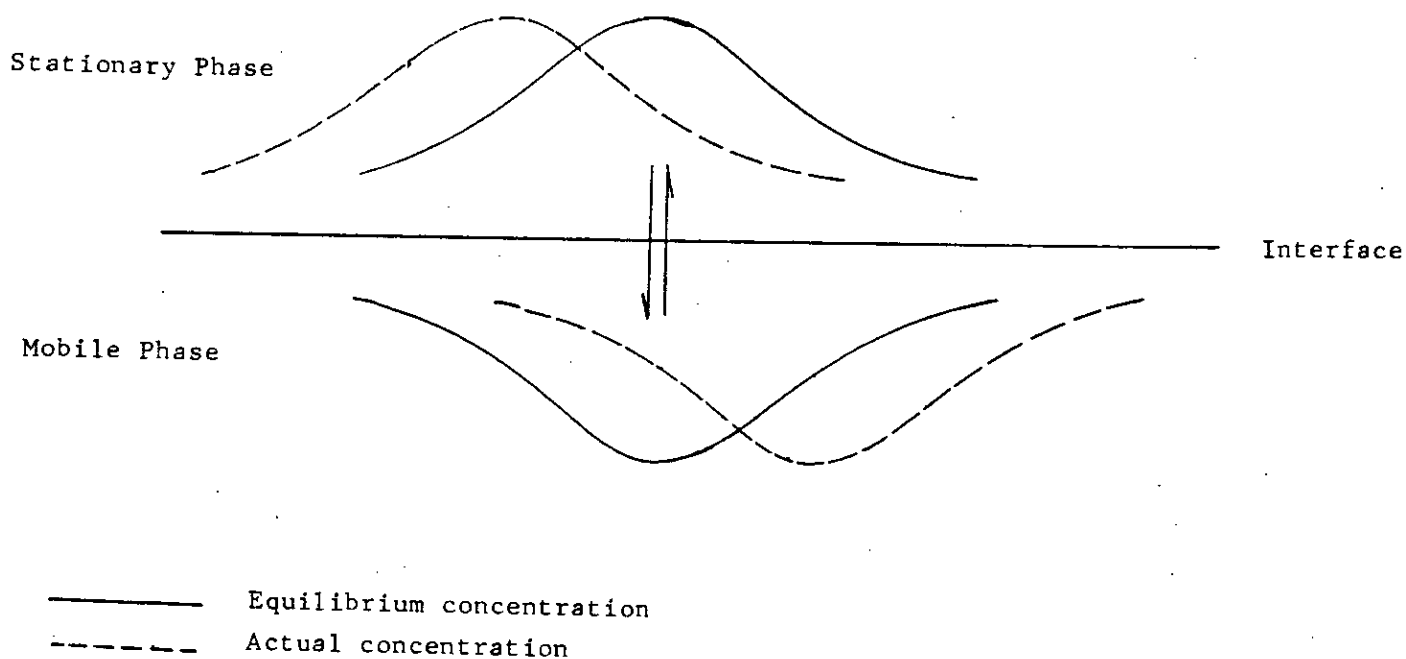
the progress of a molecule through the column is a succession of

random stops and starts about a mean equilibrium concentration. In

this dynamic non-equilibrium, represented in Figure 2-4, mass transfer

of the solute into the stationary phase results in a lag behind the

equilibrium concentration (band centre) when it desorbs and transfers

into the mobile phase the solute moves more rapidly than the band

centre. Thus dispersion increases with the number of transfers and

decreases as the velocity of the mobile phase decreases (closer

approach to equilibrium).

## 2.8 Reduced Parameters

The idea of using dimensionless (or reduced) parameters was

first developed by Giddings (13,38), has been extended by Knox and

co-workers, (25,39) and was recently reviewed by Bristow and Knox (42).

A major advantage in using the reduced or dimensionless parameters

rather than absolute parameters is that they allow ready comparison

of results obtained from columns containing packing materials of

different sizes when using eluents of different viscosities and solutes

of different diffusion coefficient. Furthermore, diverse results can

be compared from laboratory to laboratory and standards of high

performance can be easily stated and remembered.

For practical purposes there are only a few parameters which

have to be taken in account as below:

$$N = \frac{L}{H} = 16 \left(\frac{t_r}{t_w}\right)^2 \qquad \text{2-34}$$

$$h = \frac{H}{dp} = \frac{1}{16} \left(\frac{L}{dp}\right) \left(\frac{W_t}{t_r}\right)^2 \qquad \text{2-35}$$

Where h is reduced plate height and is expressed as the number of the particles in the thickness of a plate. For a good column h will be in the range of 2-10. The reduced velocity of eluent is given as:

$$\upsilon = \frac{U d_p}{D_m} = \frac{L}{t_o} \cdot \frac{d_p}{D_m} \qquad \text{2-36}$$

measures the rate of flow relative to the rate of diffusion of solute over one particle diameter. Typically $\upsilon$ will be in the range of 3 to 20 for efficient column operation.

$$l = \frac{L}{d_p} \qquad \text{2-37}$$

Where $l$ is the reduced column length.

Another important parameter is the column resistance parameter, $\varphi$, analagous to K, one of the absolute parameters, which is chromatographic column permeability and is equal to:

$$\varphi = \frac{d_p^2}{K} \qquad \text{2-38}$$

Where

$$K = \frac{U \mu L}{\Delta P} = \frac{\mu L^2}{\Delta P \cdot t} \qquad \text{2-39}$$

In equation 2-39, $\mu$ is the viscosity of eluent and $\Delta P$ is the pressure drop of the system.

Thus

$$\varphi = \frac{\Delta P \cdot t_o}{l^2 \cdot \mu} \qquad \text{2-40}$$

And the reduced plate height contribution due to column resistance parameter is equal to:

$$\frac{H^2}{K} = h^2 \varphi \qquad \text{2-41}$$

Therefore by considering the above-mentioned equations we can now express the modified Van Deemter equations with reduced parameters.

$$h = A\upsilon^{0.33} + \frac{B}{\upsilon} + C\upsilon \qquad\qquad 2\text{-}42$$

$$\quad\quad (I) \qquad\qquad (II) \qquad (III)$$

Where A, B and C are constants and factor I is expressed as the

contribution from flow in interparticle space. Despite the fact that

the eddy diffusion factor in the Van Deemter equation (2-29) was

independent of linear velocity, in the reduced plate height equation

this factor depends on reduced velocity, as shown in equation 2-42.

The exponent of $\upsilon$ (0.33) was calculated empirically by Knox (43)

from the expression:

$$h_{(flow)} = \frac{1}{a + b\upsilon^n} \qquad\qquad 2\text{-}43$$

Factor II is the contribution from axial diffusion, and finally

factor III is the contribution from slow equilibrium between mobile

and stationary phases.

It is useful to mention that the parameter A indicates the

quality of packing of a column and a value around unity shows that the

material has been well packed. A poorly packed column will have a high

value of A, say 2 to 5. The value of B was calculated and data

available in LC (44), and suggested that it is satisfactory in practice

to take B = 2. The parameter C is determined by the efficiency of mass

transfer. Values range near zero for the pellicular material, Zipax (45)

to 0.1 for materials with poor mass transfer characteristics.

## CHAPTER 3

### Thermodynamics of Liquid Chromatography

3.1    Application of different modes of LC in HPLC

The development of HPLC equipment, alongwith the improved

understanding of LC that it has generated, has drawn all these different

LC techniques together by demonstrating that all have a common theoretical

basis and can be carried out with the same basic HPLC equipment. The

modes of liquid chromatography differ only in the nature, composition,

and structure of the stationary phase, and in the nature of the

molecular forces that hold the solute molecules within the mobile and

stationary zones. A brief description of these techniques is given below.

3.2    Adsorption Chromatography

Adsorption chromatography, often referred to as liquid-solid

chromatography (LSC), is based on interactions between the solute and

adsorbent. The adsorbent is generally a porous solid with a large

reactive surface ranging from 50-400 $m^2 g^{-1}$, e.g. silica gel. Reactive

sites on this surface generally interact with the polar functional groups

of the compounds to be separated. The non-polar (hydrocarbon) portion of

a molecule has only a minor influence on the separation. Thus LSC is

well suited to separation of classes of compounds, for example, separating

alcohols from aromatic hydrocarbons. Much of our understanding of the

adsorption process is due to the work of Snyder, and a detailed account

of adsorbants and the mechanism of adsorption is given in his book (16)

which will be explained later.

Chromatographic-grade silica gels are prepared by reacting sodium

FIGURE   3-1

Surface processes occurring on heat and/or hydrothermal
treatments of silica gel: (a) Processes occurring in wide pores
(b) Processes occurring in narrow pores.

**(a)** Wide Pores



**(b)** Narrow Pores



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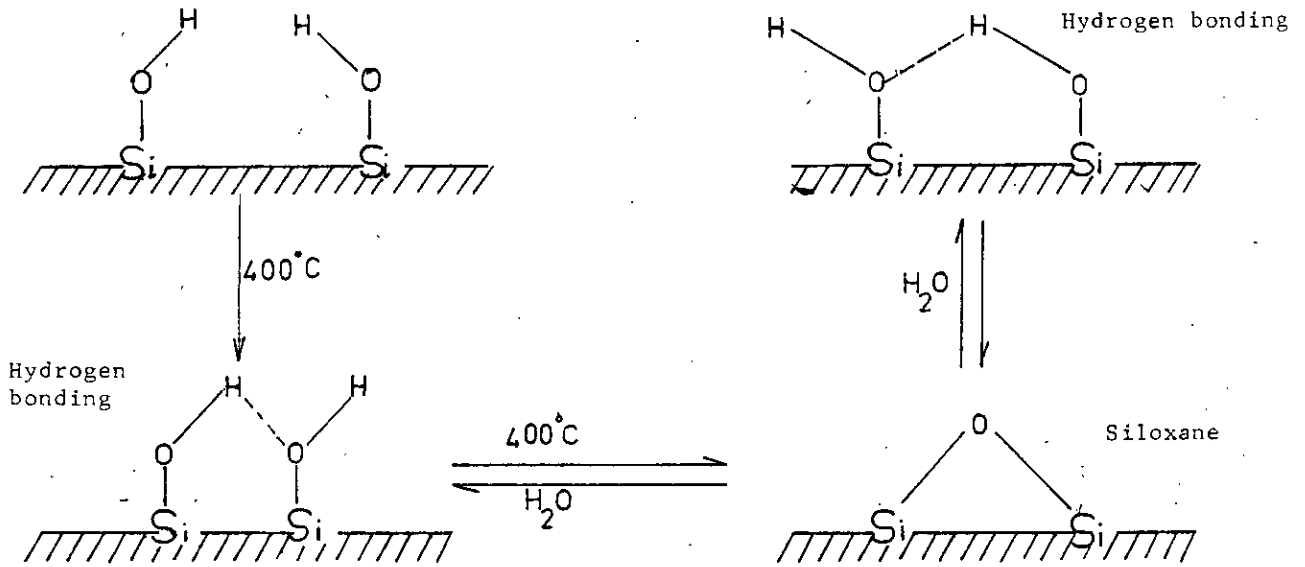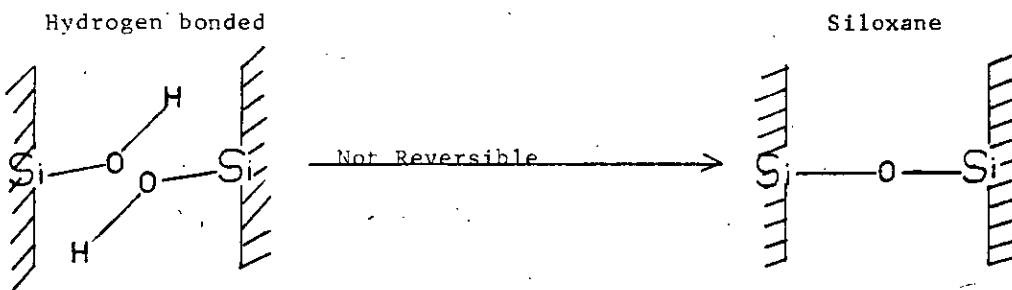
silicate with a mineral acid such as hydrochloric acid. Polymerisation occurs and a three-dimensional array of Si $O_4$ tetrahedra results.

The active sites on silica, as already mentioned, are hydroxyl groups, and therefore hydrogen bonded groups are present. The number of these depends directly on the adsorbent pore diameter. The surfaces of narrow-pore silicas are covered mainly by hydrogen bonded hydroxyls, whilst the surfaces of wide pore silicas are covered mainly by isolated hydroxyls. If the surface has not been heated above $100^{\circ}C$, many of these hydroxyls will be associated with water molecules, the majority of which can be removed by prolonged heating at $150-200^{\circ}C$. This is the normal activating procedure when using silica as a chromatographic adsorbent. Heating to temperatures greater than $400^{\circ}C$ leads to a non-reversible deactivated silica especially in narrow pore silicas, but in wide pore silicas, the reaction can be reversed by heating in the presence of water. The effects of heat on the silica surface are shown in Figure 3-1.

### 3.2.1 Thermodynamics of Adsorption

As already mentioned, the thermodynamic of adsorption has been treated in detail by Snyder (16) and a simplified version of his argument is given here.

The interaction in LSC involve a competition between the solute molecules (X), and the molecules (S) of the mobile phase for the adsorption sites. This equilibrium is illustrated by:
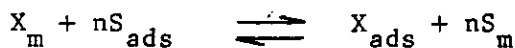
$$X_m + nS_{ads} \rightleftharpoons X_{ads} + nS_m \qquad \qquad 3-1$$

Where $X_m$ and $X_{ads}$ are the solute molecules in the mobile phase and stationary phase; and $S_m$ and $S_{ads}$ are the solvent molecules in the free mobile phase and the mobile phase molecules adsorbed on surface sites respectively. And finally, n is the number of adsorbed solvent molecules displaced by the adsorption of one molecule of X. The net energy of adsorption is given by:

$$\Delta E = E_{xa} + nE_{sm} - nE_{sa} - E_{xm} \qquad 3\text{-}2$$

Where x and s refer to the solute and solvent and a and m to the adsorbed and liquid phases.

The first simplification comes from the fact that the energy of interaction of solute and solvent in the mobile phase is very small compared to stationary phase interactions. Therefore these may be neglected, so that we can approximate equation 3-2 and write:

$$\Delta E = E_{xa} - nE_{sa} \qquad 3\text{-}3$$

$E_{xa}$ may be replaced by $S_x^o$, the sample adsorption energy on the standard activity surface, hence $E_{sa}$ may be replaced by $\varepsilon^o A_s$. Where $\varepsilon^o$ is the adsorption energy of the solvent per unit area of the standard activity surface. By convention $\varepsilon^o$ is taken as zero for pentane. Values of $\varepsilon^o$ for other solvents are then positive and conveniently fall in the range 0 to 1. $A_s$ is the area covered by solvent molecules.

Thus equation 3-3 can be derived as:

$$\Delta E = \alpha \, (S_x^o - nS_s^o) = \alpha \, (S_x^o - n. \, \varepsilon^o. A_s) \qquad 3\text{-}4$$

Where $\alpha$ is termed the surface activity of the adsorbent. When $\alpha = 1$, then $S_x^o$ and $S_s^o$ are the standard adsorption energies for the solute and solvent molecules respectively.

Finally, as already mentioned, n is the number of adsorbed solvent molecules displaced by the adsorption of one molecule of x, thus it is equal to $\frac{A_x}{A_s}$ . Therefore from equation 3-4 we have:

$$\Delta E = \alpha\ (S_x^o - A_s \cdot \varepsilon^o\ \cdot\ \frac{A_x}{A_s}\ ) = \alpha\ (S_x^o - A_x \cdot \varepsilon^o\ ) \qquad 3\text{-}5$$

In dilute solution ($n_{sa} \gg n_{xa}$ and $n_{sm} \gg n_{xm}$ ) and the thermodynamic equilibrium constant for adsorption of a sample molecule x is:

$$K_{th} = \frac{N_{xa}}{N_{xm}} \qquad 3\text{-}6$$

Where $N_{xa}$ and $N_{xm}$ are the mole fractions of x in the adsorbed and mobile phases respectively.

Therefore the mole fractions of x in two phases are:

$$N_{xa} = \frac{n_{xa}}{n_{sa}} \quad \text{and} \quad N_{xm} = \frac{n_{xm}}{n_{sm}} \qquad 3\text{-}7$$

Combining equations 3-6 and 3-7, we have:

$$K_{th} = \frac{n_{xa}}{n_{xm}}\ \cdot\ \frac{n_{sm}}{n_{sa}} = K' \cdot \frac{V_m}{V_s} \qquad 3\text{-}8$$

Where $V_s$ and $V_m$ are the volumes of stationary and mobile phases respectively.

Since $\Delta E = - \frac{\Delta G^o}{2.303RT}$ and $\log K_{th}$ is the free energy of partition:

$$\log K_{th} = \Delta E = - \frac{\Delta G^{o}}{2.303RT} = \alpha \, (S_{x}^{o} - A_{s} \, \epsilon^{o}) \qquad \text{3-9}$$

Combining equations 3-4, 3-8 and 3-9, we have:

$$\log_{10} K' = \log_{10} \left( \frac{V_{s}}{V_{m}} \right) + \alpha \, (S_{x}^{o} - A_{s} \, \epsilon^{o}) \qquad \text{3-10}$$

Equations 3-9 and 3-10 give us a remarkable description of solute

behaviour on the surface. The most important feature of this equation

is the natural emergence of an eluotropic series, that is, solvents

can be classed according to their strength of adsorption. Table 3-1

is an abbreviated eluotropic series specifically for alumina as the

adsorbent, but qualitatively this series holds for the other polar

adsorbents as well (16). An eluotropic series can be used to find an

optimum solvent strength for a particular separation. Using a solvent

of constant composition is called isocratic elution. If an isocratic

solvent is too strong (if the K' values for the solutes are too small),

a weaker solvent is substituted. Binary solvent mixtures may also be

used to find an optimum value of the solvent strength parameter $\epsilon^{o}$.

Snyder (16) suggested that in all forms of chromatography,

one must be aware of the so-called general elution problem when dealing

with isocratic solvent systems and multi component samples with widely

differing K' values. If a strong isocratic mobile phase is selected

that will adequately elute strongly remained compounds, then the weakly

retained ones will be eluted too quickly and will be poorly separated.

Conversely if a weak mobile phase is chosen, so that weakly retained

sample compounds will be separated, then very strongly retained solutes

## TABLE  3-1

### Eluotropic series for Alumina

| ELUENT | $\varepsilon^{o}$ |
|--------|--------|
| Pentane | 0.00 |
| 1-Pentane | 0.08 |
| Carbon tetrachloride | 0.18 |
| 2-chloro-2-methyl propane | 0.30  * |
| Methylene chloride | 0.42 |
| 1, 2-dichloroethane | 0.49 |
| Di-Oxan | 0.56 |
| Ethyl acetate | 0.58 |
| Diethylamine | 0.63 |
| Acetonitrile | 0.65 |
| Methanol | 0.95 |

\*   Assumed the same as for 2-chloropropane.

Reproduced by permission of Ref. (16.)

may not be eluted at all, or only very slowly, and possibly with the peaks so broadened as to be undetectable. No single isocratic solvent can be found that will be effective for such a mixture of compounds with widely K' values. To handle this kind of sample, Snyder and Kirkland (46) found that the rate of band migration must be changed during the chromatographic run. In LC the most common technique used to increase elution rates is called solvent programming or gradient elution. Here elution is begun with a weak solvent and the solvent strength is increased with time. The changes are made either stepwise or continuously.

Adsorption chromatography has been widely used for the chromatography of polycyclic aromatic compounds, phenols and amines. Since silica is acidic, amines tend to be very strongly adsorbed and may give tailed peaks, accordingly for basic compounds, alumina is usually preferred. Conversely, acidic compounds such as phenols chromatograph well on silica but not so well on alumina. Problems arise if the molecules to be separated are highly polar or contain ionizable groups in which case, poor peak shapes are almost inevitable. The problem may be reduced somewhat, for example, in the chromatography of polar acids on silica, by adding an acidic component to the mobile phase to suppress the ionization of the solutes and block the more active silanol groups, but in general reverse phase chromatography offers a more useful solution.

## 3.3    Partition Chromatography

In Partition Chromatography, also referred to as Liquid-Liquid

Chromatography (LLC), the solute molecules distribute themselves between two immiscible liquid phases, the stationary phase and the mobile phase according to their relative solubilities. The stationary phase is uniformly spread on an inert support, a porous or non-porous particulate solid. To avoid mixing of the two phases, the two partitioning liquids must differ greatly in polarity. If the stationary liquid is polar and the mobile phase is non-polar, then polar compounds are retained more strongly and this is the usual mode of operation. On the other hand, if the stationary liquid is non-polar (for instance, pentane) and the mobile phase polar (e.g. water) then polar components favour the mobile phase. The latter technique (which has reversed polarity) is referred to as Reversed Phase LLC.

The stationary and the mobile phases are selected to have little or no mutual solubility. Therefore, they generally are quite different in their solvent properties. For example, water as the stationary phase and pentane as the mobile phase for normal LLC. However, water does have a very slight solubility in pentane. If pentane used as a mobile phase is allowed to flow over a water-coated support long enough, it will slowly remove the water and change the nature of the separation mechanism. For this reason the mobile phase must be pre-saturated with the stationary phase before it enters the column. Pre-saturation can be done by stirring the two phases together until equilibration takes place, but it is more conveniently done by placing a "precolumn" before the chromatographic column (a precolumn pack should contain a high surface area packing, such as silica gel,

coated with a high percentage, say 30-40% by weight of the stationary

phase used in the analytical column). As the solvent passes through

the highly dispersed stationary phase the solvent becomes saturated

with it and will not remove it from the analytical column.

Many solvent pairs are available, and the choice of the proper

ones allows great selectivity to be achieved. Selection of useful

solvent pairs has been greatly improved by classifying solvents in

terms of their ability to undergo different types of intermolecular

interactions. Use of Hildebrand (47) solubility parameters

$(\delta_d, \delta_o, \delta_a, \delta_h)$ allows common chromatographic solvents to be classified

quantitatively in terms of parameters such as dispersion interaction,

dipole interaction, and proton donor-acceptor ability. By matching

the properties of the particular solute, with one or more of these

individual parameters, one can estimate K' values and vary $\delta$ until

the desired separation is obtained. Since the activity coefficient of

members of a homologous series vary with their molecular size, members

of an homologous series can be separated by LLC whereas in LSC there is

little discrimination between successive members of a homologous series.

3.4     Chemically Bonded Stationary Phase

This is the most common method for separation of highly polar

materials. The technique may be described as a non-polar stationary

phase in conjunction with a polar mobile phase. The polar components

now have little affinity for the hydrophobic support and are eluted

relatively quickly by the aqueous mobile phase. Since the most polar

molecules will now tend to elute first, this type of chromatography is

referred to as reversed phase chromatography.

A variety of non polar bonded phases are now commercially available. The most common of these is that formed by bonding octadecylsilyl groups ($C_{18}H_{37}Si-$) groups to silica.

The differences observed in practice with these phases can be attributed to differences in the following properties which determine chromatographic behaviour.

(1) Particle size and shape of the siliceous support.

(2) Porosity, specific surface area and pore size distribution of the silica.

(3) Chemical nature of the bonded hydrocarbonaceous moiety, eg. the chain length of the alkyl function.

(4) The amount of the bonded hydrocarbon per unit column volume, that is related to the carbon content of the dry stationary phase.

(5) The configuration of the bonded functions at the surface.

(6) The surface concentration of accessible silanol and siloxane groups.

Indeed, the longer the bonded alkyl chain, the greater is solute retention under otherwise fixed conditions, similarly, stationary phases having higher carbon content have a greater intrinsic retentive capacity. Thermodynamics of retention in reversed phase chromatography is described in Chapter 4.

3.5     Dynamically Coated Stationary Phases

        "Dynamically Coated" stationary phase chromatography is a novel

application of the ideas developed for reversed phase and ion pair

chromatography.  In this technique the hydrophilic nature of surfaces

such as polar silica or alumina is drastically modified by reaction

with a small amount of cationic, anionic, or non-ionic surfactant

dissolved in an aqueous organic mobile phase.  In this respect,

extensive studies on the adsorption of surfactants at the solid-liquid

interface and its consequences in drug preparation were recently

reviewed                        by Rupprecht (48).

He showed that in dispersions of organic solvents the adsorption of

surfactant at interfaces such as the solid $Si\ O_2$ surface is influenced

by interactions with the functional groups at the surface, adsorption

of the solvent, and by solvation of the surfactant.  Therefore

strongly polar solvents such as alcohols reduce the adsorption of

surfactant by competitive (hydrogen bonding) displacement from

adsorption sites, while in solvents like chloroform, strong solvation

reduces interactions with the solid surface.  He reported that the

highest sorption on silica was from dichloromethane and his lowest

observed surfactant adsorption was with dimethylsulfoxide (DMSO) as

solvent.  In the same paper the adsorption of non-ionic surfactants

and ionogenic surfactants at solid water interfaces was described and

the effects of added salt and variation of pH on this process were

also outlined.

        Since the silica surface is acidic, cationic surfactants

(e.g. CTAB) can be bound by electrostatic interaction; and anionic

surfactants can give the same kind of reaction with basic aluminas.

Times for complete surface equilibration depends upon a variety of

factors, as explained in the section describing chemically bonded

stationary phases, and in particular, the nature of surface and

surfactant chain length and concentration play important roles.    In

general equilibrium time varies between 1 to 2 hours (passage of 30

to 60 column volumes).  The composition of the solvent is critical

in respect to retention behaviour.   Increase in organic modifier

leads in decrease in retention (similar to chemically bonded phases).

It is particularly difficult to wash bound surfactant off the surface

of the oxide packings.

As some understanding of this technique developed, it was

applied to separation of a wide range of components from very non-

polar (e.g. naphthalene and pyrene) to very polar acidic compounds

(e.g. sulphonic acids), and also amines and amino acids and short chain

peptides.   Results were compared with those from different modes of

chromatography, and it was found that the new technique gave relative

separations similar to those on chemically bonded stationary phases.

3.6     Exclusion Chromatography

Exclusion Chromatography is also called gel-permeation (G.P.C.)

or gel-filtration chromatography.   Here the stationary phase should be

chemically inert.   Exclusion chromatography involves selective diffusion

of solute molecules into and out of mobile phase filled pores in a three

dimensional network, which may be a gel or a porous inorganic solid.

The degree of retention depends upon the size of the solute relative to the size of the pore. Small molecules will permeate the smaller pores, intermediate size molecules will permeate only part of the pores and be excluded from others, and the very large molecules will be completely excluded. The larger molecules will travel faster through the stationary phase and elute from the column first. Thus, exclusion chromatography is especially useful in separating high-molecular weight organic compounds and biopolymers from smaller molecules.

### 3.6.1 Thermodynamics of Exclusion Chromatography

To understand how G.P.C. chromatography differs from the other form of chromatography, refer to equations 2-13 and 2-14, in which:

$$V_r = V_m (1 + K') = V_m + KV_s \qquad 3-11$$

In this context, $V_m$ and $V_s$ are referred to as the void volume and the total pore volume, respectively. The distribution coefficient K depends on the molecular weight of the sample and on the pore size of the packing. In a true permeation process, assuming all pores to be accessible to a small solute molecule, $X_s = X_m$ and $K_x = 1$. If none of the pores is available to a large solute molecule (i.e. it is excluded), then $X_s = 0$ and $K_x = 0$. Intermediate molecules have access to various portions of the pore volume, for them $0 < K_x < 1$. Unlike other forms of LC, all sample molecules elute between the excluded volume Vm and the total permeation Volume $V_t$, then $V_r$ of equation 2-13 is equal to $V_t$.

Selecting the pore size of the packing depends on the size of the solute molecule to be separated as well as on the overall geometric

FIGURE 3-2

Calibration Curve for Exclusion Chromatography.

(Reproduced by permission of Ref.40)
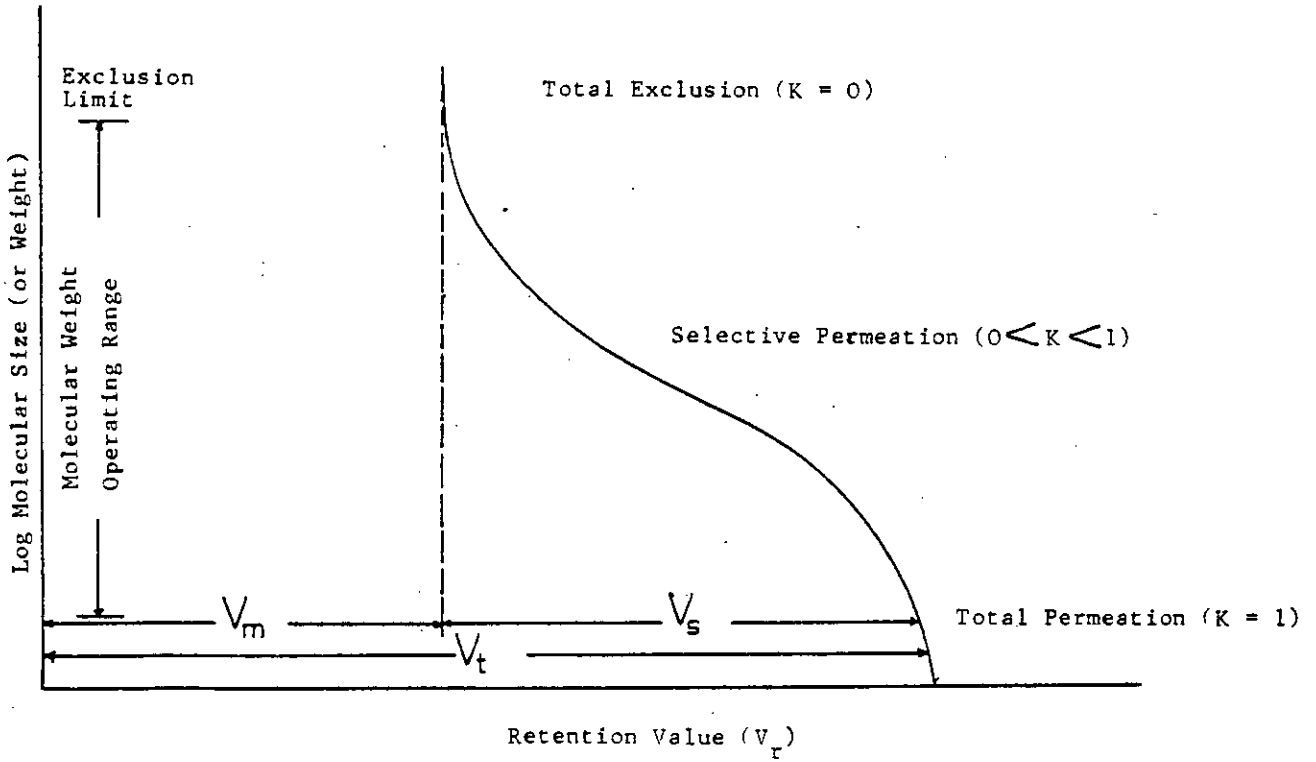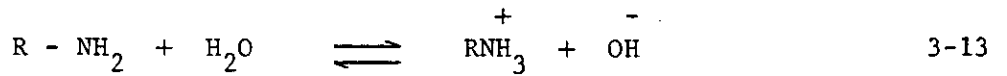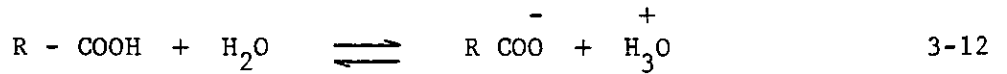
shape of the molecules (Figure 3-2). A calibration curve is usually plotted as log (Molecular Weight) versus $V_r$. Each exclusion packing of a different average pore size will have its own calibration curve. If the pore distribution is wide, the curve will have a steep slope. Thus, the molecular weight operating range will be large, but the column will provide less discrimination (resolution) of species of closely related molecular sizes. If the pore distribution is narrow, the curve will be flatter, the molecular weight operating range smaller, but the resolution of closely sized molecules will be increased.

## 3.7    Ion Exchange Chromatography

Ion exchange chromatography is based on the affinity of ions in solution for oppositely charged ions on the stationary phase. Ion exchange packings consists of a porous solid phase, usually a resin, onto which ionic groups are chemically bonded. The mobile phase is usually a buffered aqueous solution containing a counter-ion whose charge is opposite to that of the surface groups, i.e. it has the same charge as the solute, but which is in charge equilibrium with the resin in the form of an ion pair. Competition between the solute and the counter-ion for the ionic site governs chromatographic retention. Ion exchange chromatography is generally applicable to ionic compounds and to ionizable compounds such as organic acids and bases.
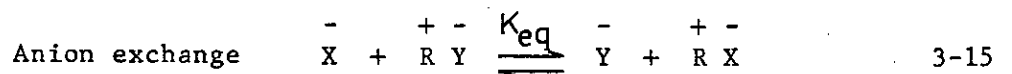
### 3.7.1 . Thermodynamics of Ion Exchange Chromatography

As already mentioned, solvents must be buffered in order to control the position of acid base equilibria:

$$R - COOH + H_2O \rightleftharpoons R\ COO^- + H_3O^+ \qquad 3\text{-}12$$

$$R - NH_2 + H_2O \rightleftharpoons RNH_3^+ + OH^- \qquad 3\text{-}13$$

If the eluent is not buffered, the proportions of the neutral and ionised forms will change throughout the chromatographic band. Since the two forms of the solute will certainly have different degrees of retention, peak tailing is inevitable.

The presence of a solute ion of the same ionic charge sets up an equilibrium as follows:

$$\text{Cation exchange} \qquad X^+ + R^-Y^+ \rightleftharpoons Y^+ + R^-X^+ \qquad 3\text{-}14$$

$$\text{Anion exchange} \qquad X^- + R^+Y^- \underset{}{\overset{K_{eq}}{\rightleftharpoons}} Y^- + R^+X^- \qquad 3\text{-}15$$

Where X is the sample ion, Y is the mobile-phase ion (counter-ion) and R is the ionic site on the exchanger.

At equilibrium, the retention is controlled by the equilibrium constant for reaction 3-15, $K_{eq}$ defined by:

$$K_{eq} = \frac{(RX^-)\ (Y)}{(X)\ (RY)} \qquad 3\text{-}16$$

$$D_{eq} \propto k' = \frac{(X_a)}{(X_m)} = \frac{(RX)}{(X)} \qquad 3\text{-}17$$

Where $X_a$ and $X_m$ are the concentrations of solute in stationary and mobile phase respectively.

Therefore, from equations 3-16 and 3-17 we have:

$$k' = K_{eq} \cdot \frac{(RY)}{(Y)} \qquad 3\text{-}18$$

Since the concentration of the ion exchange sites, $(R^+Y^-)$ is constant and fixed by the structure of the matrix, $k'$ is inversely proportional to the concentration of the counter-ion in the eluent, a variable that is easily controlled experimentally.

The pH of the eluent also effects $k'$, although only indirectly through the fraction of solute ionised. From equation 3-12 for a weak acid we have:

$$K_{dissoc.} = . \frac{(H_3^+O) (R-COO^-)_m}{(R\ COOH)_m} \qquad\qquad 3\text{-}19$$

and the capacity factor is given by:

$$k' = \frac{(RCOO)_s}{(RCOO)_m + (R\ COOH)_m} \qquad\qquad 3\text{-}20$$

Dividing the numerator of equation 3-20 by $(RCOOH)_m$ we get:

$$k' = \frac{(RCOO)_s/(RCOOH)_m}{1 + (RCOO)_m/(RCOOH)_m} = \frac{K_a}{1 + \dfrac{K_{dissoc.}}{(H_3O)}} \qquad\qquad 3\text{-}21$$

Where $K_a$ is the capacity factor of the acid when it is fully ionised and $K_{dissoc.}$ is the dissociation constant of acid. Therefore from equation 3-21 it is obvious that retention of a weak acid is increased by lowering the pH, while at pH = $pK_{dissoc.}$ value of the solute, there is 50% ionisation and therefore the capacity factor will be 0.5 times $K_a$.

CHAPTER 4

4.    Introduction and Thermodynamics of Reversed Phase and Ion
      Pair Chromatography

As pointed out in previous chapters and further shown in

investigations described in the following practical chapters, it

appears that "dynamically coated stationary phase" chromatography is

a novel form of the so-called "Soap Chromatography" technique.

Accordingly the theory of soap chromatography as well as that of

reversed phase chromatography may be applied to this new mode of

chromatography.   Therefore it is necessary to discuss in detail

the mechanism and thermodynamics of reversed phase and soap

chromatography.   These are the subject of this chapter.

4.1    Introduction, Advantages and Disadvantages of Reversed
       Phase Chromatography

The work with non polar stationary phases was first credited

to Boldingh (49) who separated long chain fatty acids on a column of

rubber powder by elution with aqueous methanol and acetone.   The

technique of Reversed Phase Chromatography (RPC), was introduced

in 1950 by Howard and Martin (29), who carried out liquid-liquid

partition chromatography on paraffin oil and n-octane as the

stationary phase using aqueous eluents.

RPC has developed spectacularly since the introduction of

chemically bonded stationary phases, first by Rossi, et al (50), in

the early 60's, who prepared benzyl and lauryl esters of silica gel

for the separation of hydrocarbons by GC.   Further development came in

the late 60's from Halasz and Sebestian (30).   New bonding techniques

have been developed and since 1974, have been applied to microparticles
of silica and alumina, so that it is now possible to prepare very
efficient columns packed with chemically bonded phases.  It was
estimated in 1977 that 60 to 80% of analytical HPLC separations were
then done by this technique.(51).

Chemically bonded stationary phases solve several problems
associated with ordinary chromatographic stationary phases.  The usual
high activity adsorbents such as silica gel, alumina, etc., tend to
separate only by chemical type.  On adsorbents with isolated high
energy sites, certain polar compounds may become chemisorbed and
labile compounds may rearrange or degrade.  Selection of liquids for
partition chromatography is not simple.  Not only must the two liquids
be sufficiently immiscible to form two stable phases, but each must
have proper solvency for all sample components.  There are frequently
mutually exclusive conditions.  Gradient elution is generally precluded
with LLC.  In both LLC and LSC, precolumns are required to equilibrate
(saturate) eluent with stationary phase, either to maintain adsorbent
modifier at the desired level, or to prevent dissolution of the
stationary liquid phase.  If a stationary phase does not remain fixed
on the solid support at the temperature of operation, column
characteristics may well change with time.  Furthermore, detector
interference may also result.  Thick liquid films generally give poor
chromatographic performance and high eluent velocities may physically
strip off a stationary liquid phase.  Friction between mobile phase
and solid support at high flow rates can cause temperature increases of a

degree or more, which may lead to increases in the mutual solubility of the two phases.

In principle, and also to a considerable extent in practice, these problems are eliminated if the stationary phase consists of organic molecules anchored by a chemical bond onto an appropriate solid support material. These stationary phases can be made mechanically, thermally, and solvolytically stable, insoluble in all but the most corrosive solvents and to possess good flow and packing characteristics. However, these supports are relatively expensive. Although they have developed rapidly in recent years, bonded phases, with a wide range of selectivities, are becoming commercially available.

4.1.1    Thermodynamics of Bonded Phases

In order to calculate the solute retention in reverse phase chromatography, one should determine the capacity factor according to free energy changes. Horvath, et al (51-53) applied the "solvophobic effect" mechanism of Sinanoglu and his co-workers (54,55), to bonded phase liquid chromatographic separations. They argued that retention of solutes could be through hydrophobic interactions between solute and non polar stationary phase rather than partitioning of solute between the non polar stationary and the aqueous mobile phase. They inferred that a monolayer hydrocarbonceous stationary phase cannot act as a bulk of non polar liquid since it is a "molecular fur" of covalently bound alkyl chains.

The capacity ratio, $K'$, is related to the thermodynamic equilibrium constant, $K$, as shown:

$$K' = \varphi \, K \qquad \qquad 4\text{-}1$$

Where $\varphi$ is the phase ratio.

The thermodynamic equilibrium constant can be expressed in term of a free energy change, therefore:

$$\ln K = -\frac{\Delta G}{RT} \qquad \qquad 4\text{-}2$$

Where $\Delta G$ is the free energy change, R is the gas constant and T is temperature.

The free energy change of the binding process arises from bringing the complex into the solvent from the stationary phase on the one hand and placing the individual components into the solvent on the other. Mathematically, this may be expressed as the sum of the two major factors.

$$\Delta G_{tot} = \Delta G_{cav} + \Delta G_{int} + RT \ln \left(\frac{RT}{P_0 V}\right) \qquad 4\text{-}3$$
$$(I) \qquad \qquad (II) \qquad \qquad (III)$$

Where an energy $\Delta G_{cav}$ is required to prepare in the solvent a cavity of size and shape suitable for the solute and $\Delta G_{int}$ measures the interaction energy between the solute and the surrounding solvent. Factor III is a "free volume" change, where $P_0$ is one atomsphere and V is the molar volume of the solvent.

We may calculate and identify individual terms:

$$\Delta G_{cav} = N \cdot A_s \cdot \gamma \, (x^e - 1) + N \cdot A \cdot \gamma \qquad 4\text{-}4$$

Where N is Avogadro's number, A is the cavity area, $\gamma$ is the surface tension of the bulk solvent, $A_s$ is the surface area of a

solvent molecule and $x^e$ is a factor which adjusts the macroscopic surface tension of the molecular dimensions.

Term II in equation 4-3 contains two distinct free energies, the Van der Waals free energy, $\Delta G_{vdw}$, and an electrostatic free energy change $\Delta G_{es}$.

$$\Delta G_{vdw} = W + a A \qquad\qquad 4\text{-}5$$

Where W and a are solvent-dependant parameters and A is the surface area of the solute molecule.

$$\Delta G_{es} = \frac{Z}{\varepsilon} \qquad\qquad 4\text{-}6$$

Where $\varepsilon$ is the dielectric constant and Z is a factor which depends on several variables. The value of Z is independant of solvent composition and is small when molecules are simple dipoles or all simple non ionized organic molecules, but it is large with ionized compounds and in general, depends on the charge distribution, molecular size and other factors (52).

Equations 4-4, 4-5 and 4-6 are dissociation free energy changes. Therefore binding free energy can be expressed as:

$$\Delta G_{cav.assoc.} = - \left[ (N.\gamma.\Delta A + N.\gamma.A_s (x^e - 1) \right] \qquad 4\text{-}7$$

Where $\Delta A = A_E + A_L - A_{EL}$ $\qquad\qquad 4\text{-}8$

and $A_E$, $A_L$, and $A_{EL}$, are the surface area of the solute, the ligand and the complex respectively.

And association energy for equation 4-5 is equal to:

$$\Delta G_{vdw.Assoc.} = - (W + a.\Delta A) \qquad 4\text{-}9$$

Where $\Delta A$ is shown in equation 4-8.

Finally:

$$\Delta G_{es.Assoc.} = - \frac{\Delta z}{\epsilon} \qquad 4\text{-}10$$

Where $\Delta z = z_L + z_E - z_{EL}$ $\qquad 4\text{-}11$

and $z_L$, $z_E$, $z_{EL}$, are the Z values of ligand, solute and complex with ligand respectively.

Now by combination of equations, 4-1, 4-2, 4-7, 4-9 and 4-10 we can express the logarithm of the capacity factor by the following relationship:

$$\ln K' = \varphi + \frac{1}{RT}\left[(\Delta A (N.\gamma + a) + N.A_s.\gamma (x^e - 1) + W - \frac{\Delta z}{\epsilon}\right] + \ln \frac{RT}{P\vartheta} \quad 4\text{-}12$$

Equation 4-12 is a simplified version of the basic equation presented by Horvath, et al (52,53). In principle, all parameters, except $\varphi$, are a function of physical properties of the solutes and eluents, and these are measurable. Therefore it can be used to explain or predict the dependance of the capacity ratio on the properties of the solutes and eluents in a given column.

## 4.2   Ion Pair Chromatography

### Introduction

The use of ion pair formation for extraction of ionizable compounds from aqueous solutions has been well developed

by Schill (56). This work was the first to apply ion pair extractive techniques to modern chromatography. He found that silica gel and cellulose could be coated with a solution of a reagent capable of forming ion pairs so that use of relatively non polar mobile phases gave excellent separations of ionic species. Before long, other workers expanded on this technique, illustrating its ability to separate a wide range of compounds such as biogenic amines, drugs and metabolites, dyestuffs, carboxylic acid, etc.

By means of ion pair chromatography, complex separations can be readily achieved. Reverse phase ion pair chromatography (RP-IPC) where one uses a hydrophobic stationary phase and aqueous buffers containing a low concentration of ion pairing reagent, allows one to separate both ionized and un-ionized components under the same chromatographic conditions. In general, by using this technique, we are able to encompass several requirements for the analysis of ionized solutes such as rapidity, sensitivity, selectivity, efficiency and an ability to resolve materials from complex systems such as biological fluids without a prior solvent extraction step.

An ion pair may be described as a coulombic association species formed between two ions of opposite electrical charge. Many variables may affect ion pair formation such as polarizability, ion constitution, and solvent dielectric constant, etc. In chromatographic separations, ion pair formations may be between inorganic-organic and organic-organic solute pairs, and the formation of each type will be highly dependant upon the immediate environments of both ions.

The theory of ion-ion association of sodium chloride in liquid ammonia was first explained by Bjerrum (57). He suggested that in solvents of low dielectric constant, ionopheres (i.e. substances composed of ions which do not combine into covalently bonded molecules) of opposite electrical charge could associate whilst still retaining their basic properties, and that the association was caused by coulombic forces and to a lesser extent by other interactions. This association of electrostatic charges leads to a net partial electrical neutrality, although it is important to understand that this is not complete and that ion pairs still have some polarity.

Then in the early 1960's, Diamond (58) realised that in aqueous solution, if both the cation and the anion are large, which generally means having some hydrophobic integrity, then there is a tendency for these large ions to be forced into a single, larger cavity by the water molecules so as to decrease the disturbance of its structure. Thus ion pairing in aqueous systems or in other highly structured (bonded) solvents is primarily enforced not by an electrostatic ion-ion interaction, but is the result of solvophobic effects which then permit ion-ion association to occur.

Our aim in an analytical context is the overall transfer of the so-called ion-ion associated complex from the aqueous environment to zones of lower dielectric constant (organic), thus the nature of the formed species and the properties of the extracting phase are essential. So $Na^{+\delta} Cl^{\delta-}$, will have a much lower oil solubility than say

$RSO_4^-$, $R_4N^+$. Ion pair formation and overall transfer are shown below:

$$A_{aq}^+ + B_{aq}^- \rightleftharpoons (A.B)_{org} \qquad 4\text{-}13$$
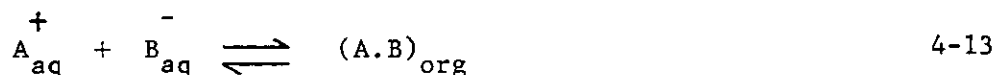
Where subscripts aq and org refer to as aqueous and organic phase respectively.

For hydrophobic ions, the formation of the ion pairs will be in the aqueous phase, followed by subsequent transfer, i.e.

$$(R\ A)_{aq}^+ + (R\ B)_{aq}^- \xrightarrow{\text{fast}} (R\ A,\ R\ B)_{aq} \xrightarrow{\text{slow}} (R.A.\ R\ B)_{org} \qquad 4\text{-}14$$
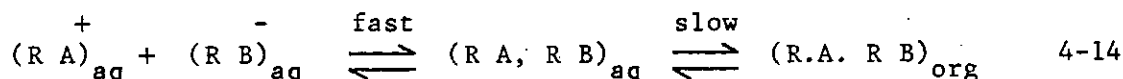
From scheme 4-14, it is assumed that R is a high molecular weight organic part of the molecule. For the species with lower molecular weight, ion pair formation will occur in the interfacial or diffusion layers between the two phases where the dielectric constant will be far lower than that of the aqueous phase.

In ion pair formation between inorganic anionic species and quaternary ammonium cationic pairing ions, it was shown (59) that the distribution ratio increases by a factor of two for each additional methylene group in the alkyl chain and it has been found further that the order of extraction of the anion is:

$$Clo_4^- > SCN^- > I^- > Clo_3^- > No_3^- > BrO_3^- > Cl^-$$
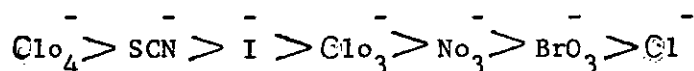
Other factors altering the distribution of the ion pair including side reactions, polarisability, and environmental factors, such as temperature, ionic strength and pH will be explained later.

## 4.2.1 Theory of Ion Pair Chromatography

If we assume that $X^-$ is the anion species of an acid and $P^+$ is the cation species of salt $P^+C^-$ and $C^-$ the counter-ion of

pairing ion $P^+$, then ion pair extraction could be expressed as:

$$P^+_{aq} + X^-_{aq} \rightleftharpoons PX_{org} \qquad 4\text{-}15$$
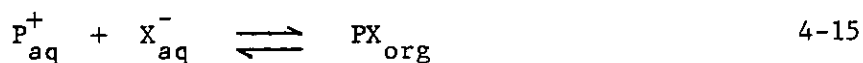
The extraction constant for equation 4-15 is equal to:

$$E_{PX} = \frac{(PX)_{org}}{(P^+)_{aq}(X^-)_{aq}} \qquad 4\text{-}16$$

The distribution coefficient $D_{PX}$ for equation 4-15 may be defined as:

$$D_{PX} = \frac{(PX)_{org}}{(X)_{aq}} \qquad 4\text{-}17$$

From the combination of equations 4-16 and 4-17 we have:

$$E_{PX} = \frac{D_{PX}}{(P^+)_{aq}} \qquad 4\text{-}18$$

Ringbom (60) improved the theory of ion pair extraction by introducing conditional factors, in which he explained that the influence of side reactions and also secondary equilibria between desired solutes and another competing species in the phase system, etc., would effect the extraction coefficient. So he introduced $E_{con.}$ (i.e. use of conditional extraction coefficient instead of extraction constant), in which:

$$E_{con.PX} = \frac{E_{PX} \cdot \alpha_{PX}}{\alpha_{X^-} \alpha_{P^+}} \qquad 4\text{-}19$$

Where $\alpha_{PX}$, $\alpha_{X^-}$, $\alpha_{P^+}$ are $\alpha$- coefficients of the side reaction for complex PX, solute $X^+$ and pairing ion $P^+$ respectively.

From the combination of equations 4-18, 4-19 then 4-20 can be written as:

$$D_{PX} = E_{con.PX} \cdot (P^+)_{aq} \qquad 4\text{-}20$$

The more practical aspect of equation 4-20 is its relationship to capacity ratio, $K'$. From equation 2-5, considering the analogy between equilibrium distribution ratio, $K_X$ and $D_{PX}$, we may write:

$$K' = \varphi \cdot E_{con.PX} \cdot (P^+)_{aq} \qquad \text{4-21}$$

Where $\varphi$ is the phase ratio $(\dfrac{V_s}{V_m})$

Equation 4-21 shows the dependence of capacity ratio on the conditional extraction constant and pairing ion concentration in reversed phase systems. For straight phase systems we may also write the same type of equations as:

$$D_{PX} = \frac{(X^-)_{aq}}{(PX)_{org}} \qquad \text{4-22}$$

and

$$D_{PX} = \frac{1}{E_{PX} \cdot (P^+)_{aq}} \qquad \text{4-23}$$

and conditional extraction constants as:

$$D_{PX} = \frac{1}{E_{con.PX} \cdot (P^+)_{aq}} \qquad \text{4-25}$$

Thus

$$K' = \varphi \cdot \frac{1}{E_{con.PX} \cdot (P^+)_{aq}} \qquad \text{4-25}$$

From equations 4-21 and 4-25, it may be shown that the relationship between $K'$ and pairing ion concentration $(P^+)_{aq}$ in straight and reversed phase systems is linear. $K'$ decreases with increasing pairing ion concentration in straight phase systems, while an increase was observed in capacity ratio with increasing pairing ion concentration when a reverse phase system was used.

Equations 4-21 and 4-25 show that the capacity ratio of an ionized solute is related to its ion extraction constant, pairing ion concentration, and the phase ratio. Thus it is important to discuss more about these parameters, especially types and characteristics of pairing ions. We could classify pairing ions into three main types, inorganic, hydrophobic, and surface active.

### 4.2.2. Inorganic Ions

As pointed out already, inorganic ions may act as pairing ions with appropriate solutes of opposite charge. One important consideration with these ions is that their high hydration will inhibit ion pairing in environments of high dielectric constant (Section 4-2), and that such association species will thus be formed in the diffusional or interfacial layers between phases. Several different inorganic ions have been used in HPLC as pairing ions such as, perchlorate, bromide, chloride and so on. The very first example of these types of separation was carried out by Wahlund and Groningsson (62), who separated some organic ammonium compounds by reversed phase partition chromatography with inorganic anions. Silicone-treated acetylated cellulose was used as the support, lipophilic alcohol mixtures as the adsorbed stationary phase, and aqueous solutions of inorganic ions as the mobile phase.

### (i) Perchlorate Ion

There are several examples of the use of perchlorate pairing ions in the literature of HPLC. For example, Borg, et al (63) separated basic analytes in a study of plasma levels.

They used cyclohexane and pentanol (93:7) for the mobile phase and an ethanolysed cellulose support coated with an aqueous phase containing Na $ClO_4$ (0.9 mol. $dm^{-3}$ in 0.1 mol. $dm^{-3}$ $HClO_4$) to give considerable selectivity of separation.

Knox and Jurand (64) used the same aqueous perchlorate solution as the stationary phase on a non-compressible stationary support, Merckosorb, SI 100 silica gel. They used halogenated hydrocarbons as eluent components with either chloroform or methylene chloride mixed with aliphatic alcohols. Cationic model solutes such as phenothiazines and dibenzazepines were separated by this system, and these workers explained such phenomena as the capacity ratio decrease with increasing percentage of alcohol in the eluent. They also found that an increase in the alcohol chain length leads to an increase in the capacity ratio (i.e. butanol to isopentyl alcohol causes a 1.4 to 2- fold increase). Substitution of chloroform by methylene chloride caused a 1.4 to 2.5 fold decrease in capacity ratio. They also discussed the effect of sample structure on retention, and showed that the central heterocyclic group of the drug's fused ring system had a major influence on retention. In the study of cationic model solutes, the order of elution was strongly influenced by the nature of the side chain attached to the central nitrogen atom. It was concluded that this ion pair chromatographic system could be most effective when a group separation is required, and that the main discriminating factor is the number and basicity of the nitrogen atoms in the compounds.

Further development of perchlorate pairing ion separation was carried out by Karger, et al, (65) who showed that better separations could be achieved by using highly solvating components diluted with hexane as the mobile phase. Good resolution of primary and secondary biogenic amine pairs was demonstrated with relatively weak solvating components such as butanol and ethyl acetate in the eluent. There are serious disadvantages in working with perchlorate ion. Firstly, bleeding or stripping of stationary phase from the support. This problem may be solved by using large surface area stationary supports, which can extract polar components from a mixed mobile phase (e.g. hexane-acetonitrile, 99:1) (66). Secondly, the problem of peak tailing. This problem can be improved by adding butanol ($\geqslant$5%) to n-hexane used as eluent (67) or by using secondary equilibria (competing ions) which will be explained later.

Subsequent work by Kraak and Huber (35) who attempted to write simple formulae to explain ion pair extraction and ion pair formation, used aqueous perchloric acid eluents and tri-n-octylamine as the stationary phase. They showed that both Kieselguhr, Merck, (5-10 $\mu$m) and low-surface area silica (Spherosil XOC 005) gave efficient separations. From a simplified version of their theory they explained the dependence of the solute distribution coefficients on single equilibria constants, pH, and ion concentration in the aqueous phase.

If one assumes distribution of acid HX between an aqueous phase (stationary phase) containing a strong acid HA (perchloric acid)

and an organic phase consisting of a long chain aliphatic amine
B (i.e. tri-n-octylamine), the following equilibria must be
considered.

(i) Liquid-Liquid distribution of the undissociated
acid HX between the stationary and the mobile phase.

$$(HX)_m \rightleftharpoons (HX)_s \qquad \text{4-26}$$
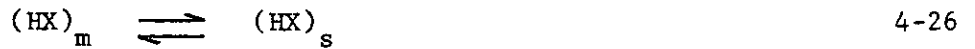
$$K_{HX} = \frac{(HX)_s}{(HX)_m} \qquad \text{4-27}$$

Where $K_{HX}$ is the liquid-liquid distribution coefficient
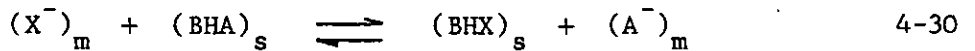of HX and subscripts, s and m refer to the stationary and mobile
phase respectively.

(ii) Dissociation of the acid HX in the mobile phase:

$$(HX)_m \rightleftharpoons (H^+)_m + (X^-)_m \qquad \text{4-28}$$
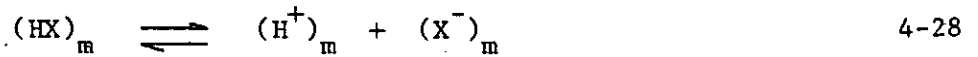
$$K_1 = \frac{(H^+)_m (X^-)_m}{(HX)_m} \qquad \text{4-29}$$

Where $K_1$ is the acid dissociation constant of HX.

(iii) Ion exchange of the anion $X^-$ in the mobile phase
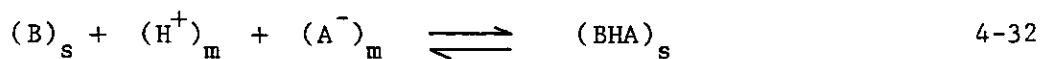with the ion pair BHA in the stationary phase.

$$(X^-)_m + (BHA)_s \rightleftharpoons (BHX)_s + (A^-)_m \qquad \text{4-30}$$

$$K_2 = \frac{(BHX)_s (A^-)_m}{(X^-)_m (BHA)_s} \qquad \text{4-31}$$

Where $K_2$ is the ion exchange constant for the anion $X^-$ in the mobile phase and the ion pair BHA in the stationary phase.

(iv) Ion-pair formation between the dissociated acid HA in the mobile phase and the amine B in the stationary phase:

$$(B)_s + (H^+)_m + (A^-)_m \; \rightleftharpoons \; (BHA)_s \qquad\qquad 4-32$$

$$K_3 = \frac{(BHA)_s}{(B)_s (H^+)_m (A^-)_m} \qquad\qquad 4-33$$

Where $K_3$ is the formation constant of the ion pair BHA in the stationary phase.

(v) Exchange of the proton of the acid HX in the stationary phase for the cation $M^+$ in the mobile phase which is assumed to be a constituent of the eluent:

$$(M^+)_m + (HX)_s \; \rightleftharpoons \; (MX)_s + (H^+)_m \qquad\qquad 4-34$$
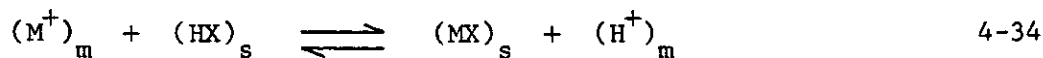
$$K_4 = \frac{(MX)_s (H^+)_m}{(HX)_s (M^+)_m} \qquad\qquad 4-35$$

Where $K_4$ is the ion exchange constant for the cation $M^+$ in the mobile phase and the acid HX in the stationary phase.

The first simplifying assumption arose from the fact that, since the sample is very dilute in the mobile phase, its concentration in the stationary phase must also be very low. Further, it is assumed that components B and BHA of the stationary phase are insoluble in the mobile phase. Under these conditions, the overall concentration of amine in the stationary phase is given by the relationship:

$$(B)_s + (BHA)_s = C \qquad\qquad 4-36$$

Where C is constant. According to equations 4-26, 4-28, 4-30, 4-32 and 4-34, the total distribution coefficient of X is given by the expression:

$$K_x = \frac{(HX)_s + (BHX)_s + (MX)_s}{(HX)_m + (X^-)_m} \qquad 4\text{-}37$$

From equations (4-26) - (4-34), an expression can be derived that describes the total distribution coefficient as the sum of three terms:

$$K_x = \Delta K_{x1} + \Delta K_{x2} + \Delta K_{x3} \qquad 4\text{-}38$$

Where

$$\Delta K_{x1} = K_{HX} \cdot \frac{1}{1 + \dfrac{k1}{(H^+)_m}} \qquad 4\text{-}39$$

$$\Delta K_{x2} = K_2 \cdot C \frac{1}{\left((A^-)_m + \dfrac{1}{K_3 \cdot (H^+)_m}\right)\left(1 + \dfrac{(H^+)_m}{K_1}\right)} \qquad 4\text{-}40$$

and

$$\Delta K_{x3} = K_{HX} \cdot K_4 \cdot \frac{(M^+)_m}{(H^+)_m + K_1} \qquad 4\text{-}41$$

Equation 4-38 describes the dependence of the total distribution coefficient upon three terms. The first term is the distribution of acid HX, the second term is the effect of ion pair formation with the amine, and the third term is the effect of ion pair formation with a monovalent cation. It also indicates that the distribution coefficient depends upon the concentrations of eluent anions, of eluent cations, and the pH of the mobile phase.

Discussion of the effect of environmental factors (i.e. pH,

salt concentration and temperature influence) on retention behaviour

by Kraak and Huber (35) showed that selectivity can be controlled by

these variables.  Effects of these variables on capacity ratio are

explained briefly here.

(a)  Effect of Temperature

In Kraak and Huber's discussion (35), the dependance

of capacity ratio on temperature was found to be insignificant in

some instances, showing small increases or decreases with temperature.

This behaviour can be explained by equation 4-38.  Choosing conditions

such that $\Delta K_{x3}$ was zero, it was found that if the first term in this

equation is dominant, then the temperature dependence of the total

distribution coefficient will be determined mainly by the partial

distribution coefficient ($K_{HX}$) of the species HX.  If the second

term is dominant, then the ion exchange constant $K_2$ will determine

the temperature dependence of the total distribution coefficient.

It can be assumed that the distribution coefficient $K_{HX}$ decreases

with temperature, whereas the ion exchange constant $K_2$ increases

with temperature.  From experimental results it has been concluded

that for phenols and carboxylic acids at very low pH (e. g. pH = 1.5)

the liquid-liquid distribution of these compounds is the major process,

because their capacity ratio decreases with temperature.  For sulphonic

acids, ion pair formation is the major process at low pH values as

the capacity ratios of these compounds increase with temperature.

A special case arises with naphtholsulphonic acids which have both

a phenolic group and a sulphonic group.  These compounds show

FIGURE  4-1

pH dependance of the capacity ratio.
Eluites: A: 2-naphthol-6-sulphonic acid;
B: 4-nitrobenzoic acid; C: Phenol. Column:
TOA, 0.04 g⃕ per gram of solid support.

(Reproduced by permission of Ref.35)
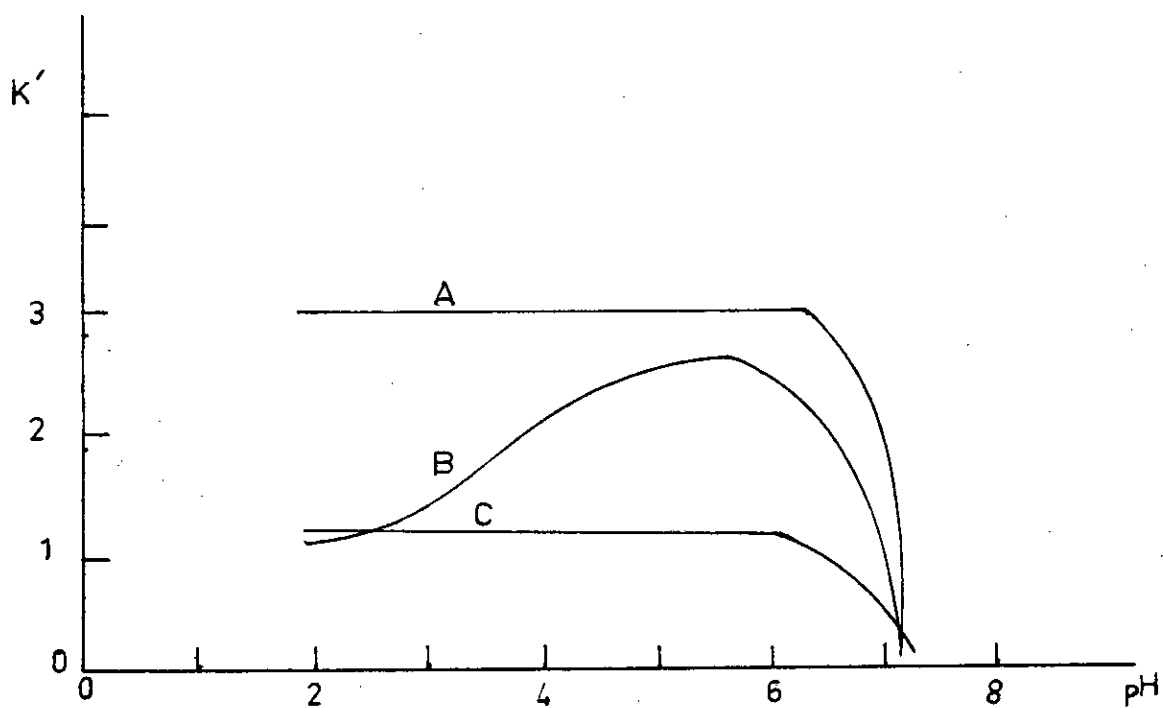
insignificant dependance of capacity ratio on temperature.

(b)  Effect of pH

The influence of pH on distribution coefficient is obvious from equation 4-38.  Briefly, from a discussion of theory and from experimental data, it was shown that the effect of ion pair formation is predominant for sulphonic acids, whereas distribution of the undissociated compounds is predominant for phenols.  In the case of carboxylic acids, both processes are significant.  For compounds with small dissociation constants, such as phenols, the capacity ratio approaches a constant value at low pH.  At higher pH values, the capacity ratio was found to decrease sharply.  For compounds with medium dissociation constants, such as carboxylic acids, the capacity ratio approaches a constant value at low pH, has a maximum value at medium pH and decreases to zero at higher pH.  For compounds with large dissociation constants, such as sulphonic acids, the capacity ratio is about constant at low pH and decreases sharply to zero at high pH.  Effects of pH on retention found by Kraak and Huber are shown in Figure 4-1.

(c)  Effect of the nature and concentration of salts

The effect of the addition of salt on the capacity ratio could be explained in two ways.  On the one hand, the anion of the salt will compete with the anion of the sample with respect to protonation and ion pair formation, while on the other hand, the cation of the salt can also form an ion pair with the anion of the sample.  As a result of the addition of a salt, one can therefore expect a change in

the total distribution coefficient if ion pair formation is involved.

The addition of sodium perchlorate (anionic salt) causes a significant decrease in the capacity ratio if a strong acid group (e.g. sulphonic acid) is present in the sample molecule. A plot of capacity ratio as a function of the reciprocal of the monovalent anion concentration gives a linear relationship. For divalent acids, a linear relationship is obtained if the total distribution coefficient is plotted as a function of the reciprocal of the square of the anion concentration. For phenols or carboxylic acids, the change in capacity ratio was insignificant. For these weak acids, the partition of the undissociated molecules must be assumed to be the predominant process. The capacity ratio changes considerably with the nature of the anion for strong acids, where the effect of ion pair formation prevails. It increases in the order of $ClO_4^- < NO_3^- < Br^- < Cl^- < HSO_4^-$. This sequence is only partially correlated with the sequence of the dissociation constants of the corresponding acids, which is $HClO_4 > HBr > HCl \approx H_2SO_4 > HNO_3$. For weak acids (phenols and carboxylic acids), where ion pair formation occurs only to a minor extent, change in the capacity ratio with the type of anion is significantly smaller. In this case the change in the activity coefficients in the aqueous phase due to the presence of different types of anions must be considered to be responsible for the change in the partition coefficients of the undissociated acids, which dominates the total distribution equilibrium.

Only minor changes in the capacity ratio result from variation of

the type of cation at constant concentration. This minor effect only

shows a uniform tendency for strong acids. The distribution coefficient

increases in the order of $Li < Na < K$. To summarise, it can be concluded

that the influence of the addition of salts, like the influence of

temperature and pH, indicates that strong ion pair formation with a

predominant influence on the total distribution coefficient occurs

only for strong acids. The distribution for weak acids is determined

by the partition of the neutral molecules and dissociation in the

aqueous phase.

### (ii)     Ion Pairing with Halide Ions

Successful separation of the quaternary emepronium ion

from its ring substituted analogues by IP-HPLC has been reported by

Groningsson, et al (68), who used silicone treated (hydrophobised)

cellulose as stationary support and pentanol as stationary phase

(pre-equilibrated with the mobile phase) and aqueous solutions of

bromide or chloride as the mobile phase. Samples were injected

initially as either the bromide or chloride salts, depending on the

mobile phase used (i.e. either $0.07$ $mol.dm^{-3}$ sodium chloride or

$0.02$ $mol.dm^{-3}$ sodium bromide). The interesting aspect of this work

was the effect of adding quantities of bromide ion into a chloride-1-

pentanol system. When a small amount of p-chloromepronium was applied

as the bromide salt, a double sample peak was observed. This was due

to column retention of both chloride and bromide ion pairs. This

effect was observed even when higher bromide concentration was used,

but only as a  peak overlapping in one broad band peak.

FIGURE 4-2

Flow diagram illustrating the technique of using a
separating and an indicating column (70), where ($B^-$)
and ($X^-$) are separating and indicating pairing ions,
respectively.

(Reproduced by permission of Ref.69)
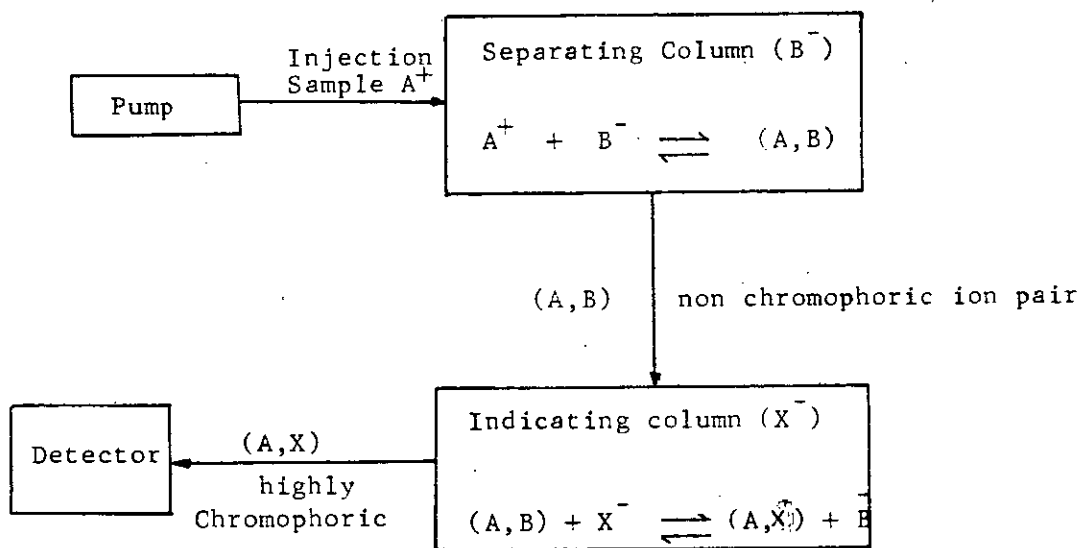
### 4.2.3    Indicating Pairing Ion

This kind of pairing ion has been used with inorganic ions such as chloride.  In other words, the inorganic pairing ions are often useless if the sample to be resolved does not absorb UV or visible light.  Eksborg (69) discussed that the separation of alkylammonium ions with chloride as pairing ion, Diachrom (particle size 37-44 µm) as a support, and chloroform 1-pentanol (19:1) as mobile phase.  Both components of the ion pairs are non-UV absorbing, thus the need of an indicating pairing ion was inevitable. He presented a method for the formation of highly U.V. adsorbing derivatives by the initial isolation of a non-chromophoric sample as an ion pair with chloride in a separating column, which is then exchanged for a highly U.V. absorbing ion in a small column (i.e. the indicating column) situated between the outlet from the separating column and an inlet to the detector.  In Eksborg's work, naphthalene-2-sulphonic acid was used as indicating pairing ion.  This method is shown schematically in Figure 4-2.

### 4.2.4    Secondary Equilibria Suppression

That peak  asymmetry with IP-HPLC is a major drawback to this technique is a significant problem has been observed by several groups.  Different variables could give rise in this problem.  Firstly it could be caused by dissociation or dimerization of the pairing ions in the organic phase.  The degree of dissociation may vary with the nature of the ion pair (70).  This effect can be suppressed by using

appropriate combinations of pairing ions. Secondly, dissociation or association processes of migrating samples may give rise to peak asymmetry. This phenomenon will vary with the concentration of sample. Eksborg, et al (71) explained that small amounts of sample sometimes gives rise to leading peaks. Thirdly, dissociation will also vary with the polarity of organic phase, e.g. with methylene chloride and pentanol, resulting in a higher ion pair dissociation than with chloroform and hexane (72). The disturbances caused by ion pair dissociation increase when the sample concentration is low. Eksborg, et al (70, 71) showed that the effect of secondary equilibria can be completely suppressed by adding excess hydrophobic ions to the organic phase.

The following expressions can be given for picrate pairing ion as an example. If the mobile phase contains ion pairs of picrate $(P^-)$ and solute ion $(Y^+)$ and with addition of excess amount of hydrophobic ion $(X^+)$, then the following equations can be written:

$$(P)_m = (X)_m + (Y)_m \qquad \qquad 4\text{-}42$$

$$(PX) \rightleftharpoons (P)_m + (X)_m \qquad \qquad 4\text{-}43$$
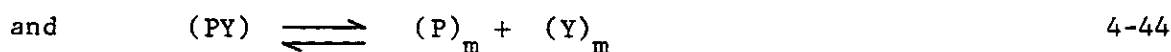
and $$(PY) \rightleftharpoons (P)_m + (Y)_m \qquad \qquad 4\text{-}44$$

From 4-43 and 4-44 we have:

$$K_{diss.(PX)} = \frac{(P)_m \cdot (X)_m}{(PX)} \qquad \qquad 4\text{-}45$$

and
$$K_{diss.(PY)} = \frac{(P)m\ (Y)m}{(PY)m}$$
4-46

and also
$$E_{PX} = \frac{(PX)}{(P)\ .\ (X)}$$
4-47

$$E_{PY} = \frac{(PY)}{(P)\ .\ (Y)}$$
4-48

Where $K_{diss(PX)}$ and $K_{diss(PY)}$ are the dissociation constants of complex (PX) and (PY) and $E_{PX}$ and $E_{PY}$ are extraction coefficients of complexes PX and PY respectively.

We now return to section 4.2.1, particularly equation 4-19, in which we explained that the $\alpha$ - coefficient depends upon association, dissociation and the reaction processes. A conditional extraction coefficient, $E_{con.}$ was explained with reference to $\alpha$ - coefficients. Thus, secondary equilibrium dissociation is one $\alpha$ - coefficient process and can be expressed as:

$$\alpha_{PX} = \frac{(PX)m + (X)m}{(PX)m} = 1 + \frac{K_{diss.(PX)}}{(P)m}$$
4-49

From the combination of equations (4-42) - (4-49), the concentration of pairing ion in the mobile phase can be written as: (88)

$$(P)_m = \sqrt{K_{diss(PX)}\ .\ E_{(PX)}\ .\ (P)\ (X) + K_{diss(PY)}.\ E_{PY}\ (P).\ (Y^+)}$$
4-50

and a simplified version of equation 4-50 can be expressed as:

$$(P)_m = \sqrt{(a\ [Y] + b)\ .\ (P)}$$
4-51

Where a and b are constant.

Explanation of this phenomena in normal phase may be seen in equation 4-51. If it is presumed that a $(Y) \gg b$, then pairing ion concentration will vary with sample concentration. Further assumptions that the pairing ion has a greater influence on $\alpha_{PX}$ and therefore on K' and that the sample concentration will decrease during the migration process suggests that the capacity ratio will also decrease with decrease in sample concentration. But on the other hand, if $b \gg a$ (Y), then the process may be easily controlled by variation of the concentration of hydrophobic ion $(X^+)$. Thus it is possible to overcome this phenomena by sufficiently increasing the concentration of hydrophobic ions and thereby to produce symmetrical and reproducible peaks.

Similar expressions for reverse phase systems were given by Wahlund (73), who assumed that if pairing ion concentration $(P^+)$ was kept at a constant level in the presence of sufficient added hydrophobic ion $(X^-)$, (much greater than sample anion concentration), and further that the dissociation constants for PX and PY are similar, then $E_{con.(PY)}$ (equation 4-19) will be independent of sample concentration and can be expressed as:

$$E_{con.(PY)} = E_{PY} \cdot (1 + K_{diss.(PY)} \cdot \left[ K_{diss.(PX)} \cdot E_{PX} \cdot (P^+) \cdot (X^-) \right]^{-\frac{1}{2}}$$

4-52

It is obvious from equation 4-52 that if the concentrations of pairing ion and the excess added hydrophobic ion are kept constant, then $E_{con.(PY)}$ will be independent of sample ion concentrations and this situation should lead to symmetrical and reproducible peaks.

4.2.5    Hydrophobic Ions

Separations by this type of ion pair chromatography generally require an aqueous mobile phase and an organic solvent stationary phase loaded on either conventional silica or alkyl-bonded silica support. The most efficient and selective separations can be achieved with chemically bonded stationary phases made with different types and lengths of organic coating.  Several different methods have been suggested for coating stationary phases on to porous silica, the most facile being the "in situ" method, in which the stationary liquid phase is applied to the packed column by means of adsorption from the mobile phase.  The different types of hydrophobic ions are explained below:

(i)    Small*tetra*Alkylammonium Ions

The advantages of using *tetra* alkylammonium ions as pairing agents were first explained by Wittmer, et al (75) and Schill, et al, (56, 74), who showed that these ions are aprotic and may therefore be used in all values.  However, this fact can be a disadvantage because the solvent must always be buffered to obtain the desired pH. An advantage arises from the fact that, since they are both hydrophobic and ionised, they will be able to form water structure-enforced ion pairs in environments having a high dielectric constant (58).  This tendency will be reduced by decreasing the number of methylene groups in their alkyl chains (reducing hydrophobicity).  Thus capacity ratio can be manipulated by controlling the hydrophobicity of the pairing ion. Schill, et al (74), showed that the capacity ratio increases by about 0.5 log unit for each methylene group added to the hydrophobic part of

an*tetra*alkylammonium ion. Moreover, increasing the number of methylene
groups in the hydrophobic structure of the pairing ion also increases
the oil-water partition coefficient, and therefore the solubility of
the formed ion pair decreases in the mobile phase, so that an increase
in hydrophobicity of the solvent (organic modifier)becomes necessary.

The question of using straight - or reversed - phase technique
was not clearly established in early studies of this type of
chromatography. There are two major advantages of using reversed-phase
rather than straight phase. Firstly, because the alkylammonium ion in
reversed-phase chromatography is present in the mobile phase, selectivity
(and capacity ratio) can be adjusted by controlling pairing and counter
ion concentrations in the mobile phase. Secondly, Wahlund (73) suggested
that it is possible to improve separations by the use of gradient elution
systems and by direct injection of large sample volumes. Such approaches
with straight phase systems lead to column instability.

One of the major considerations in the use of alkylammonium
pairing ions is the nature of any aliphatic alcohols added to the eluent.
Different workers introduced different aliphatic alcohols, for example,
Schill and his co-workers (56, 70, 71) preferred pentanol as stationary
phase in reversed phase systems and pentanol-chloroform mixtures as the
mobile phase with straight phase systems. This alcohol has the advantage
of both the extracting ability and different range of $E_{PX}$ value required
for either straight or reversed phase systems. However, pentanol does
give rise to side reactions of dissociation of ion pairs in ⌐‾⌐ polar
solvent compositions. Wahlund (73) calculated conditional extraction
constants for this system as:
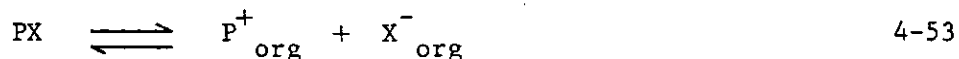
$$PX \; \rightleftharpoons \; P^{+}_{\;org} \; + \; X^{-}_{\;org} \qquad\qquad 4\text{-}53$$

From the combination of equations 4-49, 4-53 and 4-19, we have:

$$E_{con.(PX)} \; = \; E_{PX} \; \cdot \; \alpha_{PX} \; = \; E_{PX} \; \cdot \; (1 + \frac{K_{dissoc.(PX)}}{(P^{+})_{org}} ) \qquad 4\text{-}54$$

Where

$$K_{dissoc.(PX)} \; = \; \frac{(P^{+})_{org}(X^{-})_{org}}{(PX)} \qquad\qquad 4\text{-}55$$

This effect can be eliminated by using secondary equilibria suppression, which was explained in Section 4.2.4.

Our knowledge of the effects of environmental parameters on this mode of IP-HPLC is incomplete and very few workers have investigated the effects of temperature or ionic strength in alkylammonium ion pairing systems. One brief report on the effect of salt concentration was produced by Su, et al (76) who examined the effect of salt concentration on sulphonamides using tetrabutylammonium pairing ions in a straight phase system with 1-butanol-heptane as mobile phase. They found that the capacity ratio decreased by a factor of two to three as the ionic strength was approximately doubled. They also pointed out that selectivity of separation depends on the ionisation of the eluite. In the same paper they examined the effect of pairing ion concentration on retention. Double log plots of capacity ratio versus the reciprocal of tetrabutylammonium ion concentration showed a linear relationship for completely ionised sulphonamides.

In a similar study, Wahlund (73), explained that the capacity ratio of the hydrophobic ion pair formed between toluenesulphonate

and tetrabutylammonium rises from zero to 28 in a reversed phase

system with pentanol as the stationary phase, when the concentration

of pairing ion changes from zero to 0.05 mol.dm$^{-3}$.  Observations were

not supported by the work of Fransson, et al (77) on pairing between

4-hydroxybenzoate and tetrabutylammonium ions, in which non-linear

relationships were found.  Explanation of these differences requires

careful consideration of side reactions in calculations of $E_{con.(PX)}$.

### (ii) Picrate Ions

In the previous investigations of ion pair formation

with alkylammonium ions, it was observed that anionic species can

be paired with cationic pairing ions and separated successfully by

optimisation of the operating conditions.  Separation of cationic

species such as amines, amino acids, short peptides and quaternary

ammonium ions by IP-HPLC, call for anionic pairing agents instead of

cationic pairing ions.  Picrate ion and its analogues have been used

for this purpose.

Since picric acid is a strong acid, it is capable of forming

ion pairs with many cationic solutes.  Several separations of

different types of amine have been reported using picrate ion pairs.

For example, Eksborg and Schill (56, 70) separated quaternary

ammonium ions by pairing with picrate ion which had been pre-loaded

onto an ethanolysed cellulose support at pH 11.2 using chloroform/1-

pentanol as the mobile phase.  The cellulose support was used to

avoid the dissolution reactions of silica and bonded silicas at such

high pHs (2 $<$ silica pH $<$ 8).  This work was followed by Frei and his

co-workers (78, 79), who applied the principles outlined by Schill's

group, and achieved separation of some plant alkaloids paired with

picrate ion on rigid stationary support particles.  Separation of

alkaloids hyoscyamine, scopolamine and an ergot alkaloid in three

minutes was reported in their paper.  They used picric acid dissolved

in a picrate buffer as stationary phase, and 0.01 to 0.03 mol.dm$^{-3}$ of

picrate ion concentration increases due to the effects described by

the equation 4-25.  Furthermore, as the pH of the media was increased,

the concentration of protonated alkaloids decreased, which led to

higher capacity ratios.  A major advantage of picrate ions is their

intense absorption of UV light, which allowed sensitive detection of

very poorly absorbing alkaloids (Ca  500X increase in sensitivity).

These workers also pointed out that the technique is non-destructive

and can be used for preparative purposes.

Some of these amine analytes may not be readily protonated

and are distributed in neutral form, therefore it should be considered

that total distribution may also vary.  An expression similar to

equation 4-25 can be written for total distribution when straight

phase picrate ion pairing was used:

$$D = \frac{1}{E_{con.(PXH)} \cdot (P^-)_s} + D_x \qquad\qquad 4\text{-}56$$

Where $(P^-)_s$ represents the concentration of picrate ion in the

stationary phase and $D_x$ is the distribution of neutral (non-protonated)

amine, and (X) is the concentration of neutral amine.  The separation

conditions are usually chosen so that the distribution of unionized

amine will be zero or very small. This condition may be achieved by increasing pH to the alkaline range, which usually causes the problem of picrate ion bleeding from the stationary phase. A second problem arises from the high dissociation constant of picrate ion pairs, but this problem may also be eliminated by using secondary equilibria suppression as explained in Section 4.2.4.

### (iii) Alkylsulphonate Ions

It was pointed out above that both short and long chain alkylammonium ions may be used in ion pairing separations of anionic species. In the same manner, alkylsulphonates can be used as anionic pairing ions for separation of cationic analytes. Brown and his group. (80, 81) have reported on this type of ion pair HPLC. Reversed phase systems have usually been applied with alkylsulphonate ion pairs, which are commercially available in quite a wide range of hydrophobicity.

### 4.2.6. Specific Pairing Ions

A major advantage of ion pair HPLC is its flexibility in separations of a wide variety of solutes, particularly in its ease of control selectivity by variation of eluent system parameters. There are two major problems which must be taken into account. Firstly, separation and identification of solutes, giving a low detector response and secondly, separation of samples in nanogram levels. The above-mentioned pairing ions are useful for general purposes, but to overcome different problems, we cannot necessarily apply "routine" pairing ions to all purposes. For example, picrate ion pairing systems give a high detector response and may be used for sensitive analysis
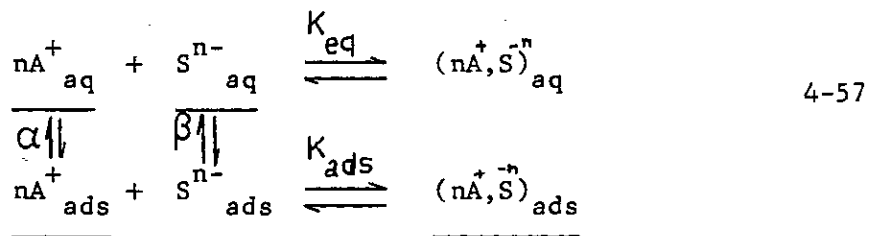
of solutes of low detector response (70, 78, 79).

4.2.7   Surface Active Ions

In Section 4.2 it was shown that ion pair formation of solute and pairing ion depends strongly on the nature (electrostatic) of pairing salts and the dielectric constant of the solvent.  In other words, if it is assumed that solvents such as water (high dielectric constant) are used as eluent bases, it is almost impossible to use chloride pairing ion with any solute, because hydrogen bonding with chloride ion is so strong that ion pair formation is impossible in this media.  Thus dissociation of ion pairs in a high dielectric solvent was reported by several groups who often found effects of tailing, fronting, peak position variability and (especially with straight phase systems) column instability.

Knox and Laird (36) examined possible improvements of column efficiency in HPLC by means of ion pair chromatography. They attempted separations of some sulphonic acids by straight phase chromatography.  However problems arose from the fact that the strong sulphonic acid was displacing water from the silica surface and peak tailing resulted.  They assumed that this problem might be resolved by employing a strong pairing ion which might be expected to form highly stable ion pairs which would not readily dissociate. Accordingly, they used a $1\%$ solution of CTAB to prevent irreversible adsorption of sulphonic acids on the polar adsorbent silica surface. These workers also used the same pairing ion with a more polar eluent to separate the same sulphonic acids on a bonded stationary phase,

SAS silica. Excellent resolution was achieved in these studies in which the authors referred to their new technique as "soap chromatography." Knox and Laird explained that by adding small amounts of CTAB (up to $5.5 \times 10^{-2}$ mol.dm$^{-3}$) to the aqueous eluent, sulphonated dyestuff intermediates can be paired with hexadecyltrimethylammonium ion and partitioned between an organic stationary phase and an aqueous eluent. The phenomenon of partition may~~$\wedge$~~ be ~~understood by~~ three physicochemical processes, firstly, formation of a CTAB sample ion pair in the aqueous mobile phase and distribution of the paired complex into the hydrophobic stationary phase; secondly, adsorption of CTAB/via hydrophobic interactions onto the surface of the reversed phase support and consequent ion exchange between the counter-ion and the solute in the stationary phase, and thirdly, partitioning of a pairing ion-counter ion complex into the hydrophobic stationary phase. They present these equilibria by equation 4-57.

$$
\begin{array}{ccccc}
nA^{+}_{aq} & + & S^{n-}_{aq} & \xrightleftharpoons{K_{eq}} & (nA^{+},S^{-n})_{aq} \\
\alpha\Updownarrow & & \beta\Updownarrow & & \\
\underline{nA^{+}_{ads}} & + & \underline{S^{n-}_{ads}} & \xrightleftharpoons{K_{ads}} & \underline{(nA^{+},S^{-n})_{ads}}
\end{array}
\qquad 4\text{-}57
$$

Where the subscripts aq and ads refer to the eluent and adsorbed phases, respectively and A and S are cetyltrimethylammonium and sample ions respectively, and where underlined species are considered to be present in high relative concentrations. Knox and Laird (36) showed in this paper that measurement of break through volumes of the pairing ion may be used to determine adsorption of CTAB by the surface.

The adsorption isotherm was curved over a $0-5 \times 10^{-2}$ mol.dm$^{-3}$ CTAB

concentration range and apparently obeyed a simple Freundlich-type

equation, i.e.

$$(A^+)_{ads} = \alpha \, (A^+)_{aq}^{0.8} \qquad\qquad 4\text{-}58$$

Taking equation 4-58 into account suggested that at $5 \times 10^{-2}$ mol.dm$^{-3}$

CTAB concentration using their support material (Wolfson SAS silica),

surface coverage of CTAB cation was between 5 and 15%, depending on

the "depth" assigned to the alkyl chain on the surface. They

suggested that such a low surface coverage is an indication that the

surface was already covered with aliphatic alcohol molecules from the

mobile phase. To relate equation 4-57 to sample retention and capacity

ratio they assumed that the concentration of sample ion $(S^{n-})_{ads}$

in the stationary phase is negligible compared to that of the A,S

ion pair and it can be derived that the distribution coefficient, D,

for a sample between the stationary and eluent phase is:

$$D = \frac{\alpha^n \cdot \beta \cdot K_{ads} \, (A^+)_{aq}^{0.8n}}{1 + K_{aq} \cdot (A+)_{aq}^n} \qquad\qquad 4\text{-}59$$

Where K is the formation constant of the (A,S) ion pair in each phase.

As is shown in equation 4-59, distribution increases with CTAB

concentration, so capacity ratio should also increase, but at higher

concentrations the capacity ratio will fall. The decrease in the

capacity ratio at high CTAB concentrations was explained as a

consequence of micelle formation of CTAB or at least as CTAB ion

clusters which solubilised the sample ions. It could also be
explained that as the concentration of CTAB increases, the excess
CTAB can hydrophobically interact with CTAB already adsorbed at
the surface and so alter the hydrophobic nature of the surface as
to cause the decrease in capacity ratio.

Knox and Laird in the same paper, examined the dependence
of dyestuff and intermediate capacity ratios on CTAB concentration,
on a silica surface using straight phase chromatography and they
found that the above observation cannot be seen with this type of
chromatography. The observed trend is for the degree of retention
to increase with increase in CTAB concentration, although the rate
of increase falls at high surface active agent concentrations.
These workers concluded from many experimental observations that
since no evidence of pairing ion adsorption on silica gel was found,
therefore, sample retention with straight phase systems using surface
active pairing ions, is one in which (A,S) ion pairs are present in
the eluent and are solvated by the aliphatic alcohol (1-propanol)
present. These solvated ion pairs are then adsorbed most readily
onto a surface which can itself be heavily solvated by 1-propanol,
so that the 1-propanol molecules act as a "binder" to hold lipophilic
ion pairs onto the hydrophilic silanol surface. Also there is no
involvement of micellar or clustered CTAB species.

Similar investigations on separations of cationic
catecholamines were reported by Knox and Jurand (82) and Jurand (83).
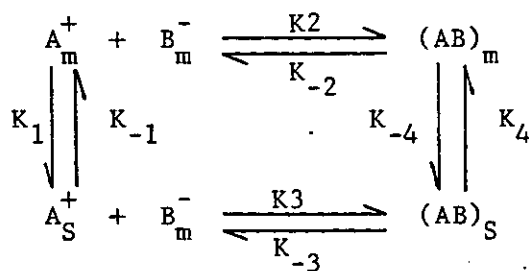In these papers, long chain anionic surface active materials, such as

sodium-dodecyl sulphate, sodium dodecylsulphonate and sodium dodecylbenzenesulphonate were used. Similar relationships between capacity ratios and concentrations of surface active materials as CTAB were reported with the 0 to $3 \times 10^{-2}$ mol.dm$^{-3}$ concentration range of alkyl sulphonate, but the order of sample elution was approximately reversed from that found using straight phase adsorption and perchlorate ion pair HPLC.

Tomlinson, et al (84) examined the effect of different chain lengths in the retention of model tryptophan solutes. They explained that three effects can be seen when alkyl chain length changes. Firstly, there is an increase in capacity ratio of tryptophan with increasing pairing ion concentration; secondly, for an equivalent concentration the capacity ratio increases with the size of the alkyl chain of the pairing ion and thirdly, there is a sigmoidal relationship between capacity ratio and pairing ion concentration.

The theory and mechanism of retention by reversed phase IP-HPLC were discussed at length in a series of papers by Horvath and his co-workers (53, 85), in which they introduced the "solvophobic" theory of IP-HPLC. In this discussion (53), they suggested that the retention mechanism in soap chromatography is primarily dependent on ion pair formation in the mobile phase, so that increasing ease of formation of the ion pair results in increase in retention. In the same paper, they argued that it is possible to partition the ion paired complex between non polar bonded stationary phase (octadecyl silica) and neat aqueous solvent by adjusting the pH of the media.

Separations of acidic and basic catecholamine metabolites and
mandelic acid derivatives were accomplished by elution from columns
packed with Partisil 10 ⬭ ODS with aqueous sodium sulphate
(1.0 mol.dm$^{-3}$) solution containing phosphate buffer (0.1 mol.dm$^{-3}$,
pH 2.1 for catecholamine metabolites and pH 4.4, for mandelic
acid derivatives).  In a further study (85), cationic species
(amino acids and short peptides), were separated on Lichrosorb
RP-18 columns with an aqueous eluent (0.1 mol.dm$^{-3}$) phosphate
buffer containing 3 x 10$^{-3}$ mol.dm$^{-3}$ sodium dodecylsulphate).

Selectivity and versatility of surface active pairing agents
stimulated many research groups to investigate the application of
different types of surfactants to several general and specific
purposes.  Tomlinson, et al (86) introduced the use of alkylbenzyl-
dimethylammonium chlorides (ABDAC'S) in the concentration range of
1 x 10$^{-5}$ — 6 x 10$^{-4}$ mol.dm$^{-3}$ for separation of the anti-allergy
drug, sodium cromoglycate, in aqueous solutions and urine.  These
workers calculated the amount of hydrophobic adsorption of surfactant
on the non polar surface by a colorimetric method and explained the
mechanism of retention as the sum of four different processes:

$$
\begin{array}{ccccc}
A_m^+ + B_m^- & \underset{K_{-2}}{\overset{K2}{\rightleftharpoons}} & (AB)_m & & \\
K_1 \big\downarrow\big\uparrow K_{-1} & & K_{-4}\big\downarrow\big\uparrow K_4 & & \\
A_S^+ + B_m^- & \underset{K_{-3}}{\overset{K3}{\rightleftharpoons}} & (AB)_S & &
\end{array}
\qquad 4\text{-}60
$$

Where subscripts S and m refer to the stationary and mobile phases

respectively and $(A^+)$, is the concentration of surface active agent,

and $(B^-)$ is the concentration of solute molecules. From the above

equations they further explained that two prime mechanisms dominate

solute retention, one is ion pair formation in aqueous phase $(K_2)$

and then partition of paired complex into the stationary phase $(K_4)$

and second is the hydrophobic interaction between surfactant and

surface $(K_1)$ and ion exchange between counter ion and surfactant

and solute molecule $(K_3)$.

As discussed above, possible mechanisms of solute retention

in soap chromatography, as first outlined by Knox and Laird (36),

followed by the solvophobic theory of Horvath, et al (53, 85), and

latterly by Tomlinson (86), were all based on hydrophobic interactions

between the surfactant and a non polar surface, but Kraak, et al (87)

argued that the interaction strictly depends upon the adsorption

isotherm. In their work, small amounts of anionic surfactant

(sodium dodecylsulphate, sodium dodecylsulphonate or dinonylnaphthalene-

:sulphonic acid) were added to aqueous eluents and the resulting systems

acted as conventional ion exchange systems. This group showed that

the degree of retention of a series of amino acids depends upon the

nature and concentration of anionic surfactant, temperature,

concentration and type of organic modifier, hydrophobic support, the

pH, and finally, concentration of counter ions in the eluent. The

theoretical consideration of distribution coefficients outlined in

their work showed that ion pair interaction is dependent upon the pH

and counter ion in one hand and two pKa values of amino acids on the other hand. They argued that counter ion concentration and temperature are important parameters, and they explained that if other parameters are kept constant, there should be a linear relationship between the concentration of the counter ion and the reciprocal of the capacity ratio. Which was indeed shown in their paper (87). This group also pointed out that an increase in temperature will cause a decrease in the capacity ratio, but, unlike other workers who have explained that increase in temperature leads to loss of water from the hydrophobic surface and consequent reduction of retention, Kraak argued that increasing temperature reduces the value of the ion exchange coefficient and hence capacity ratio.

In conclusion, the mechanism of solute retention by ion pair formation is still the subject of argument. Kraak, et al (87) showed that the concentration of counter ion is yet another parameter which may be altered to control sample retention and elution.

More recent attempts to develop new types of surface active materials and recent application of so-called ion pair techniques (i.e. using inorganic and hydrophobic ions) will be briefly discussed in the sequel.

CHAPTER 5

Ion Pair Reversed Phase HPLC of Sulphonic Acids
and Reversed Phase Chromatography of Aromatic Hydrocarbons
by Solvent Generated Hydrophobic Chromatography

5.1     Introduction

Ion Pair techniques for chromatographic resolution of mixtures
of ionised solutes were discussed in the previous Chapter.  Difficulties
with analyses of aromatic sulphonic acids, industrial intermediates of
considerable significance in dye manufacture, arises from their high
polarity, low volatility, and thermal instability which makes
separation by GC impossible without prior derivatisation.

Although there are reports of LC separations of sulphonic acids
on silica and cellulose supports by thin layer chromatography (89) and
paper chromatography (90), all of these suffered from poor separation
efficiency.  More satisfactory separations of aromatic sulphonic acid
were shown by Schmit and Henry (91), who used anion exchange
chromatography on Zipax SAX.  This report showed that although separation
by 40 μm Zipax is more than adequate, column efficiency is very poor.

On columns packed with microparticulate silica and eluted by
an aqueous/organic (water: methylene chloride: methanol) solvent toluene
could be eluted as a zone equivalent to 1700 theoretical plates, but
sulphonic acids gave broad zones of only 130 theoretical plates.  The
sulphonic acids were apparently so polar that they compete with water
for the active sites on the silica surface.

The solution of this problem lay in the use of ion-pair chromatographic techniques as originated by Horvath and Lipsky (92) and Eksborg and Schill (70) in which a relatively low concentration of a charged solute (e.g. alkylsulphonic acid or alkylammonium salt) in the solvent allows formation of hydrophobic ion pairs with the analytes. Hydrophobic interaction of these ion pairs with the alkyl chains of a bonded silica packing surface provided a means of separation of polar analytes.

The use of long hydrophobic chain surfactants with covalently bonded phase by Knox and Laird (36), opened a new page in ion pair chromatography as outlined in Chapter 4. This approach was followed in an investigation of the chromatographic properties of porous ceria microspheres by Gilbert and Wall (93) who used ion pair partition chromatographic separation of some quaternary ammonium ions and aromatic sulphonic acids as a means of probing the surface properties of this packing material. In this study an aqueous solution (picrate buffer, pH 6.7) was the stationary phase and organic mobile phase (2% pentanol: chloroform) was used to elute quaternary ammonium analytes as their picrate ion pairs in liquid-liquid partition system. The second part of this paper applied "soap chromatography" of Knox and Laird to the separation of sulphonic acids on ceria microspheres. Methanol:Water (60:40 $^v/v$) containing less than 1% CTAB was found to be a suitable eluent and gave results similar to those reported by Knox and Laird (36). These workers also pointed out that as the pH of the media is increased, retention of sulphonic acids is decreased, an effect

previously reported by Kraak and Huber (35) and discussed in Chapter 4. Gilbert and Wall (93) showed that soap chromatography provides a useful probe of the relative hydrophobicity of column packing surfaces.

Following the logic of this earlier work on "soap chromatography", columns of porous zirconium oxide gel and silica gel were packed and tested as adsorbent HPLC column packings. Then the interaction of conventional porous silica gel packings with quaternary ammonium salt surfactants were re-examined. The procedures were repeated several times and it soon became obvious that dissolved surfactant reacts with reactive oxide surfaces to produce a new packing-solvent interface which behaves very much as do "bonded phase" column packings.

This "Solvent Generated Hydrophobic Surface" was used for HPLC separations of a number of non-polar and polar analytes. Apparently, many analyses which had previously been done on surface-modified column packings could have been accomplished with equal efficiency using conventional oxide gel packing materials without prior chemical modification.

5.2    Proposed Solute Retention Mechanism

Solute retention in this mode of LC can be envisaged as occurring in two steps. In the first step the silica surface is dynamically equilibrated with surfactant dissolved in the mobile phase.
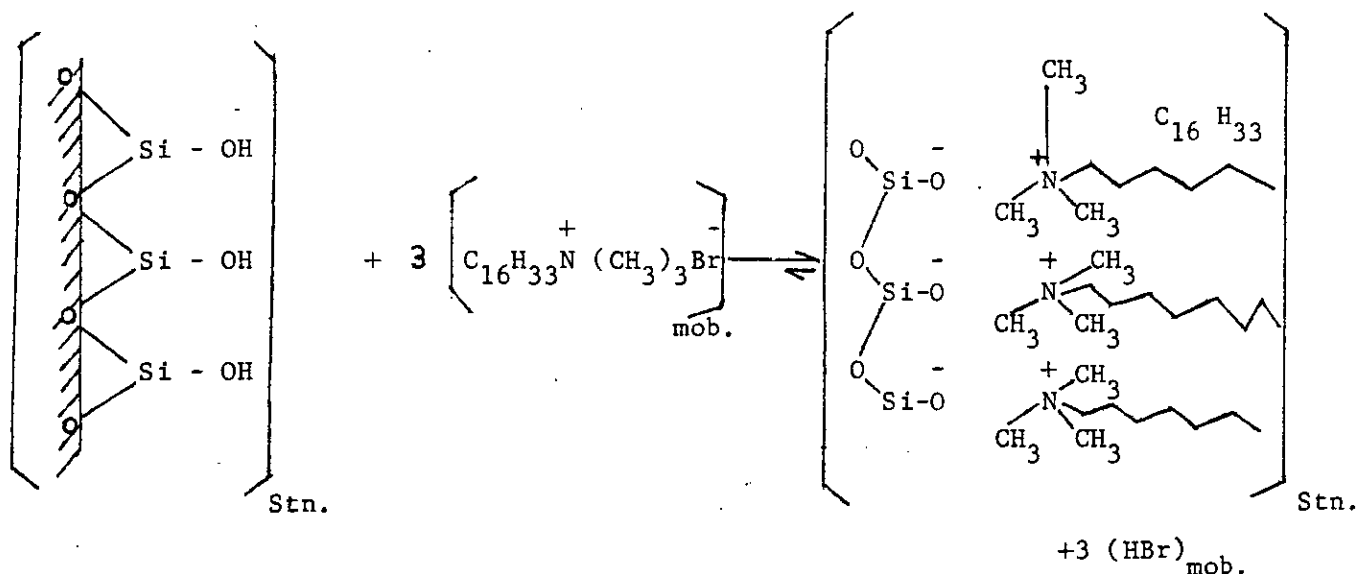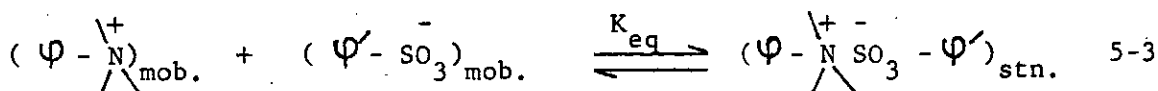
## Scheme 5-1

Scheme 5-1 shows that surface coverage increases with surfactant concentration. In other words, the more surfactant present in the solvent mixture, the greater the surface coverage.

The second step can be expressed as two processes. Firstly, surfactant (CTAB) can be ionised in the mobile phase and then ion pair formation between ionised sulphonic acids (analytes) and CTAB can occur in the stationary phase. Mechanism can be expressed as:

$$( \varphi - \overset{+}{N} Br)_{mob.} \rightleftharpoons ( \varphi - \overset{+}{N})_{mob.} + (Br)^{-}_{mob.} \qquad 5\text{-}1$$

$$( \varphi' - \overset{-}{SO_3}\overset{+}{H})_{mob.} \rightleftharpoons ( \varphi' - SO_3)^{-}_{mob.} + (H)^{+}_{mob.} \qquad 5\text{-}2$$
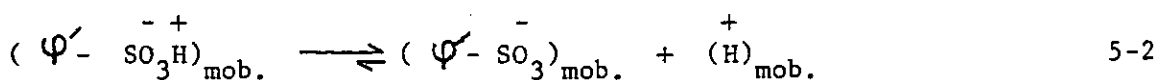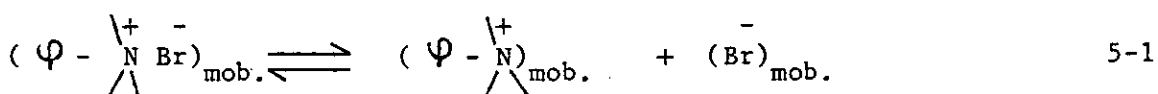
Sulphonic acid analytes may be assumed to be 100% ionised in the solution at all pH's greater than ~1.5, then:

$$( \varphi - \overset{+}{N})_{mob.} + ( \varphi' - SO_3)^{-}_{mob.} \overset{K_{eq}}{\rightleftharpoons} ( \varphi - \overset{+}{N} \, \overset{-}{SO_3} - \varphi')_{stn.} \qquad 5\text{-}3$$

Therefore

$$K_{eq} = \frac{(\varphi - \overset{+}{N} \, SO_3 - \varphi')_{stn}}{(\varphi - \overset{+}{N})_{mob.} \, (SO_3 - \varphi')_{mob.}} \qquad 5\text{-}4$$

Where $\varphi$ and $\varphi'$ are the lipophilic portions of CTAB and the sulphonic

acid analytes respectively, and mob. and stn. are indicated as mobile

and stationary phases respectively and $K_{eq}$ is the equilibrium constant

of equation 5-3.

Capacity ratio can be expressed as:

$$K' \propto D = const. \frac{(X)_{stn}}{(X)_{mob}} = const. \frac{(\varphi - \overset{+}{N} SO_3 \varphi')_{stn}}{(\varphi' - SO_3^-)_{mob}}$$

$$= const. K_{eq} \cdot (\varphi - \overset{+}{N})_{mob} \qquad\qquad 5-5$$

Where D is the distribution coefficient and $(X)_{stn}$ and $(X)_{mob}$ indicate

the concentration of solute in stationary and mobile phase respectively

and const. includes the phase ratio, which is the ratio of the volumes

of the mobile and stationary phases.

   Equation 5-5 shows that at low concentration of CTAB the capacity

ratio is proportional to the concentration of CTAB in the mobile phase.

Therefore, retention increases with increasing surfactant concentration,

but the above mentioned mechanism does not hold when the concentration

is very high.  In the second process it is assumed that the surface

carries a nearly complete monolayer of the surfactant and, accordingly,

increasing surfactant concentration from this point on leads to double

layer formation on the surface.  In other words, hydrophobic interaction

between excess dissolved surfactant and pre-equilibrated surface can occur.

The result of this process may be a change of retention mechanism from

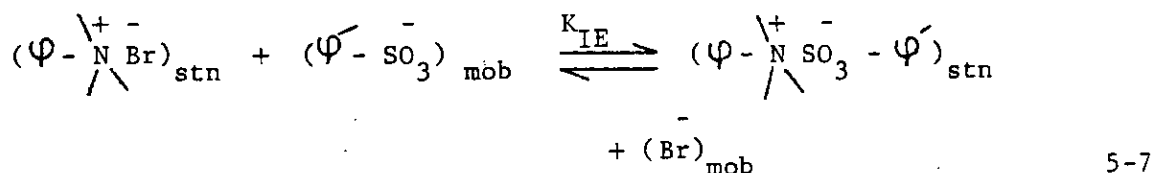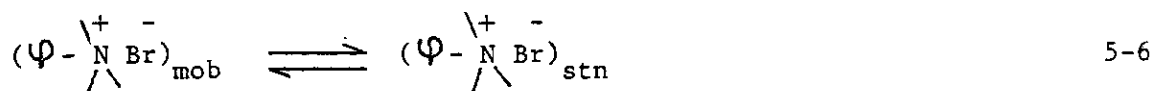ion pair partition to ion exchange with the  bilayer stationary phase

incorporating other processes (such as increase in solvation characteristics of the eluent) which will be discussed later in detail.

When the process changes from ion pair partition to ion exchange, analyte capacity ratios should eventually decrease due to the effects of the consequent increase in counter ion concentration on the ion exchange process.
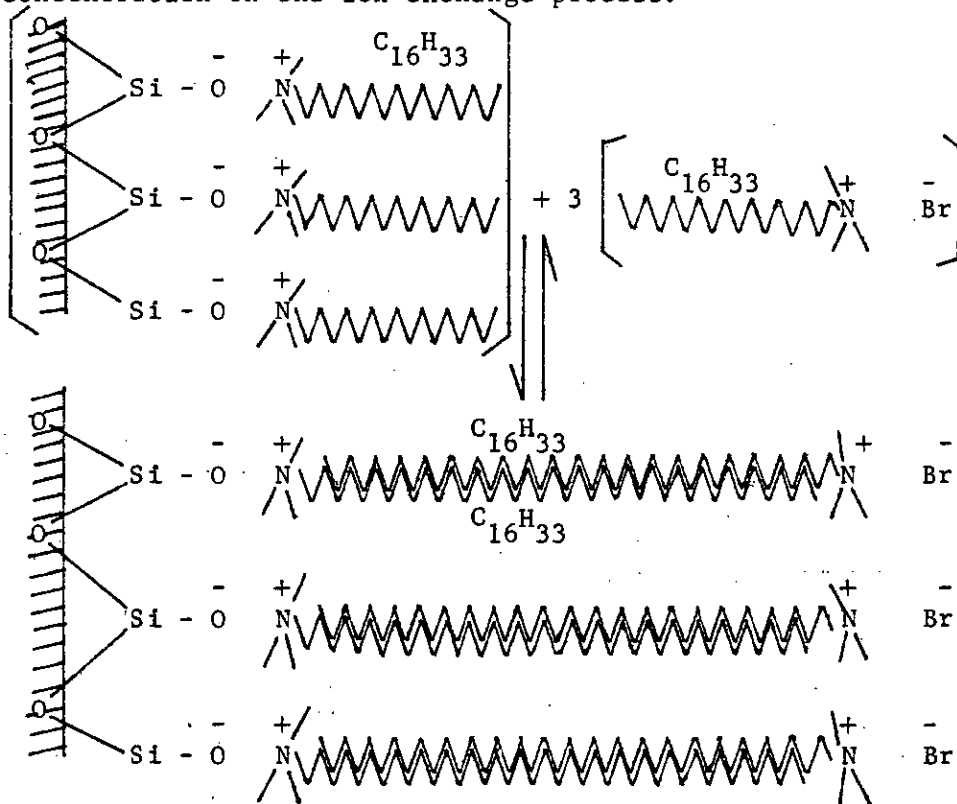


Scheme 5-2

Scheme 5-2 shows that the surface is no longer quite as hydrophobic as the original monolayer but has polar character.  Therefore, a proposed mechanism can be written as:

$$(\varphi - \overset{+}{N} Br)_{mob} \rightleftharpoons (\varphi - \overset{+}{N} Br)_{stn} \qquad 5\text{-}6$$

$$(\varphi - \overset{+}{N} Br)_{stn} + (\varphi' - SO_3^-)_{mob} \xrightarrow{K_{IE}} (\varphi - \overset{+}{N} SO_3^- - \varphi')_{stn}$$

$$+ (Br^-)_{mob} \qquad 5\text{-}7$$

Thus

$$K_{IE} = \frac{(Br^-)_{mob} (\varphi - \overset{+}{N} SO_3^- - \varphi')_{stn}}{(\varphi' - SO_3^-)_{mob} (\varphi - \overset{+}{N} Br)_{stn}} \qquad 5\text{-}8$$

and

$$K' \propto D = const. \frac{(\varphi - \overset{+}{N} SO_3^- - \varphi')_{stn}}{(\varphi' - SO_3^-)_{mob}} = const. K_{IE} \frac{(\varphi - \overset{+}{N} Br)_{stn}}{(Br^-)_{mob}} \qquad 5\text{-}9$$

Equation 5-9 shows that at a fixed (constant) concentration of CTAB the amount of $(\varphi - \overset{+}{N} Br)_{stn}$ in this equation stays constant in the stationary phase and therefore, capacity ratios should be inversely proportional to the concentration of counter anion $(Br^-)$ in the mobile phase. But the concentration of CTAB was varied in our experiments, and accordingly the concentrations of both counter anion and (CTAB) vary during experiments. The ratio of $\dfrac{(\varphi - \overset{+}{N} Br)_{stn}}{(Br^-)_{mob}}$ from equation 5-9 shows that this ratio could be constant if the amount of CTAB on the surface is directly and linearly proportional to the solution concentration. But the isotherm is curved (36), and presumably eventually reaches a constant unchanging plateau value. $(Br^-)_{mob}$ would still increase, hence K' would decrease with further increase in (CTAB). The experimental evidence which justifies this argument is incomplete, but the results
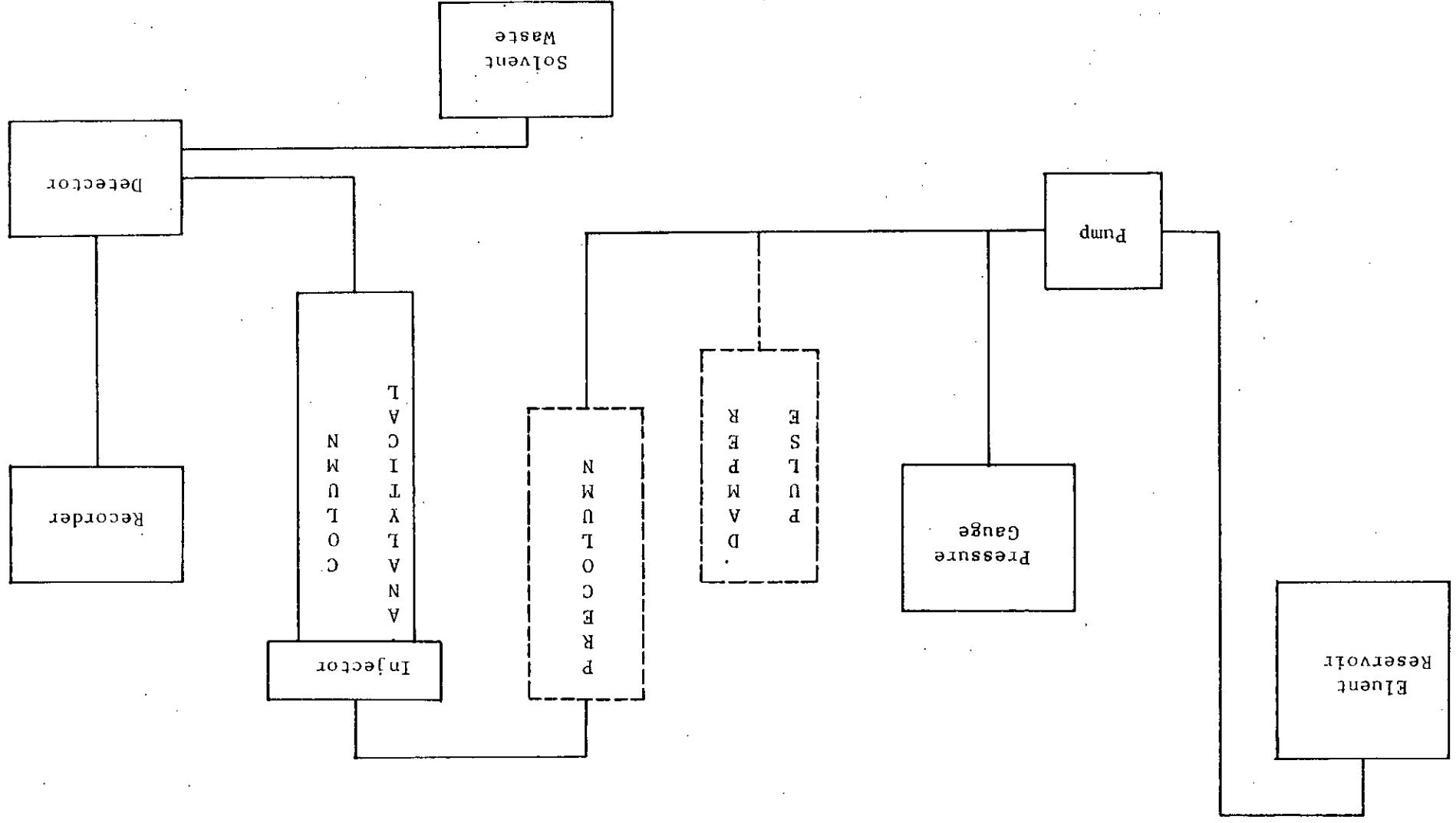
Solvent Waste

Detector

Pump

Recorder

ANALYTICAL COLUMN

Injector

PRECOLUMN

PULSE DAMPER

Pressure Gauge

Eluent Reservoir

Essential features of a Liquid Chromatograph

FIGURE 5-1

of Chapter 6 shows that at high concentration of surfactant (at least
in single surfactant systems), the mechanism of solute retention may
not be primarily ion exchange. At these higher CTAB concentrations
aggregates or "pseudo micelles" may very well be present in the mobile
phase which would significantly affect the balance of solvating power
of the eluent and hydrophobicity of the dynamically coated stationary
phase and hence reduce analyte retention.

5.3    Equipments and Experimental Techniques

The essential features of a high pressure liquid chromatograph
are indicated in Figure 5-1. The main components are:

(1)   The solvent reservoir

(2)   The high pressure pump

(3)   The analytical column

(4)   The detector

(5)   The electronics to amplify and record the detector
      signal.

In addition, the following two features may be necessary:

(6)   When a single head reciprocating pump is used, a device
      to damp out pressure pulsations may be required.

(7)   If liquid-liquid partition chromatography is employed
      then a pre-column is necessary to ensure that the
      mobile phase is saturated with the stationary phase.

(i) · Eluent Pumping Systems

The pumps used in our experiments were:

(a)   A single head reciprocating pump (Altex Model 110
      solvent metering pump).

(b) The dual head reciprocating pump (Waters Associates
Model 6000 A Solvent Delivery System).

(c) A Haskell Pressure Intensifier Pump (Haskell Engineering
and Supply Co.) Model MCP-71. This pump was used for
column packing.

## 5.3.1  Detectors

In all experimental work reported here ultra-violet photometric
detection was used. This is the most popular HPLC detector since it
combines relatively high sensitivity with ease of operation and is
capable (with variable wavelength) of detecting most samples likely
to be encountered. The maximum sensitivity of a typical ultra violet
detector is 0.01 absorbance unit full scale deflection (with 1% noise).
With this sensitivity it is possible to detect concentrations as low
as $mol.1.^{-1}$ for a compound with an extinction coefficient of $10^4$.
The ultra violet detectors used were:

(a) Cecil Instruments Model CE 212 Photometer.
This detector has variable wavelength device
and can operate between 220-400 nm.

(b) Applied Chromatography Systems (Fixed Wavelength
at 254 nm)  UV detector Model 750/10a.

All solvent line connections detectors, pumps and columns
were made with PTFE or stainless steel tubing and Swagelok or Waters
couplings.

The detector output was recorded on Servoscribe IS potentiometric
chart recorders.

FIGURE  5-2

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Outline designs for column inlet fittings, showing syringe
needle in the injection position.  Fixing nuts are omitted.



(a)                     5 or 10 μm                    (b)
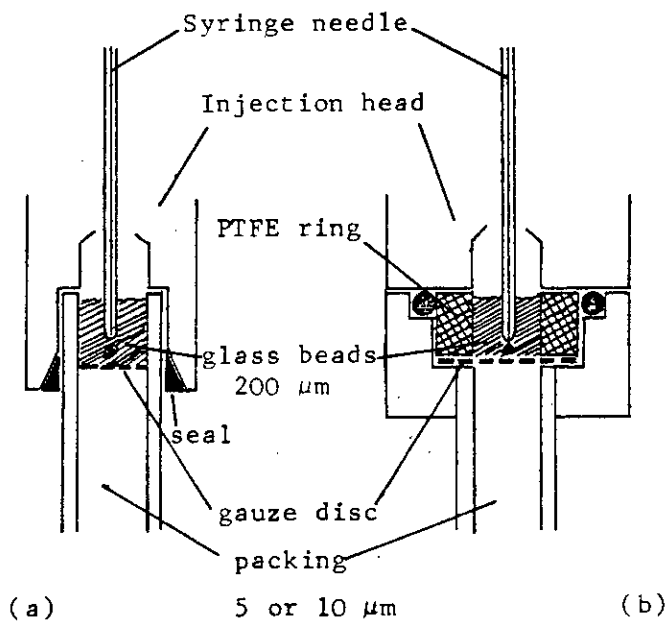
### 5.3.2 Column, Injector, and Packing Materials

One of the most important aspects of use of an HPLC instrument system is the optimisation of column parameters such as particle size and surface area, column packing technique, column length and diameter, and injection systems. In these experiments all columns were made from Apollo grade (Accles and Pollock, Oldbury, Warley, G.B.) polished (4.6 mm diameter) bore AISI316 grade stainless steel tubing in lengths of either 100 or 125 mm.

Sample introduction may either be by a loop or by direct injection from a syringe. Loop injectors are very reproducible and hence useful for quantitative work, but they may allow an increase in zone dispersion at the top of the column. Direct syringe injection systems allow on-column sample introduction so that a minimum contribution is made to sample zone variance at the inlet point. The column-injector system used is shown schematically in Figure 5-2.

### 5.3.3 Column Packing

In order to achieve the optimum Kinetic performance in HPLC, it is vital to be able to pack columns reproducibly. HPLC columns are usually packed with microparticles whose Stokes' equivalent diameters fall in the range from 3 to 20 $\mu$m. A typical batch of commercial HPLC packing material should have 95% of its particles within a two-fold range of particle diameter centred on the nominal size. Particles below 20 $\mu$m in diameter are difficult to pack by a dry packing (i.e. rotate, bounce and tap) method (94), widely used for particles of 20 $\mu$m and above. As a consequence slurry packing techniques have been widely used. These were

FIGURE 5-3

---

Details of packing chamber and column



Inlet fitting

Fixing nut

O-ring

Packing chamber

Column fixing nut
O-ring
PTFE ring
Shelf

Column

Gauze
O-ring

Tail fitting
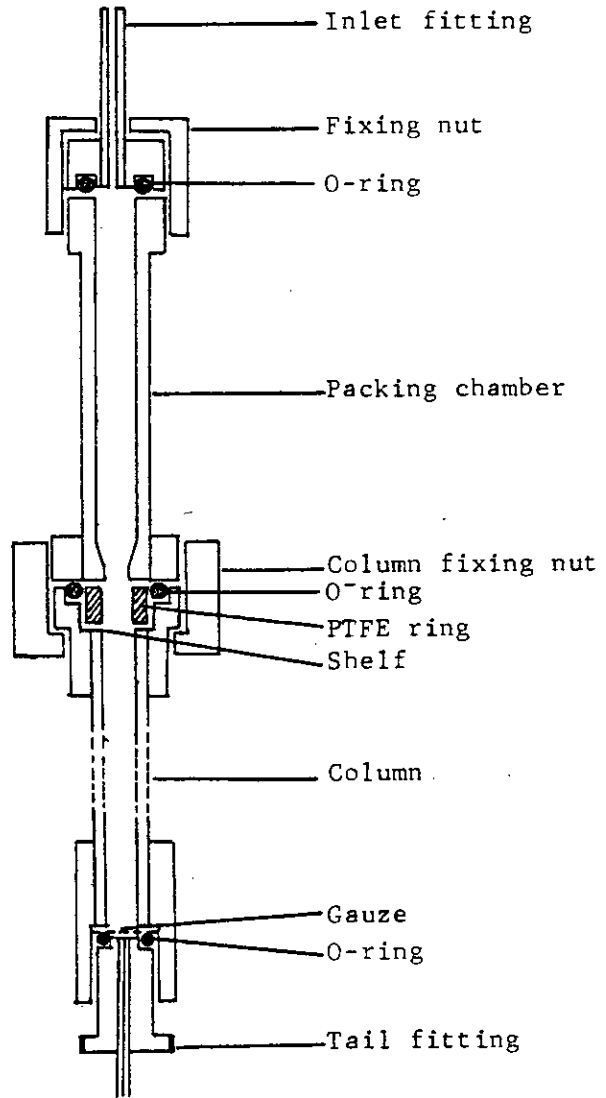
reviewed by Bristow (28), who suggested that there are five requirements for satisfactory slurry packing, namely:

(1) The particles must not sediment too fast during the procedure.

(2) The particles must not agglomerate.

(3) The particles must hit the accumulating bed at a high impact velocity.

(4) Each particle should have time to settle in before it is buried by other particles landing on top and finally,

(5) The liquid used to support the slurry must be easily washed out of the packing and must not react with it.

Bristow largely resolved all the above requirements by packing columns with slurry pumped against the pull of gravity. In this orientation sedimentation is relatively unimportant and might indeed even be beneficial in removing aggregates and large particles. He also observed by packing glass tubes that the growing surface of the bed remains perfectly horizontal when packed from below but becomes domed when packed in a descending mode. He found that methanol was an excellent supporting liquid for silica and alumina packing materials. An outline of the packing chamber used in this present work is shown in Figure 5-3.

The columns used in this laboratory were packed by the upward-flow slurry packing technique at ca 300 bar liquid pressure. Silica, zirconia, and alumina were suspended and packed in methanol. Alkyl bonded silicas were suspended and packed by the same method using methanol as suspending solvent.

FIGURE 5-4

FRACTIONATION APPARATUS
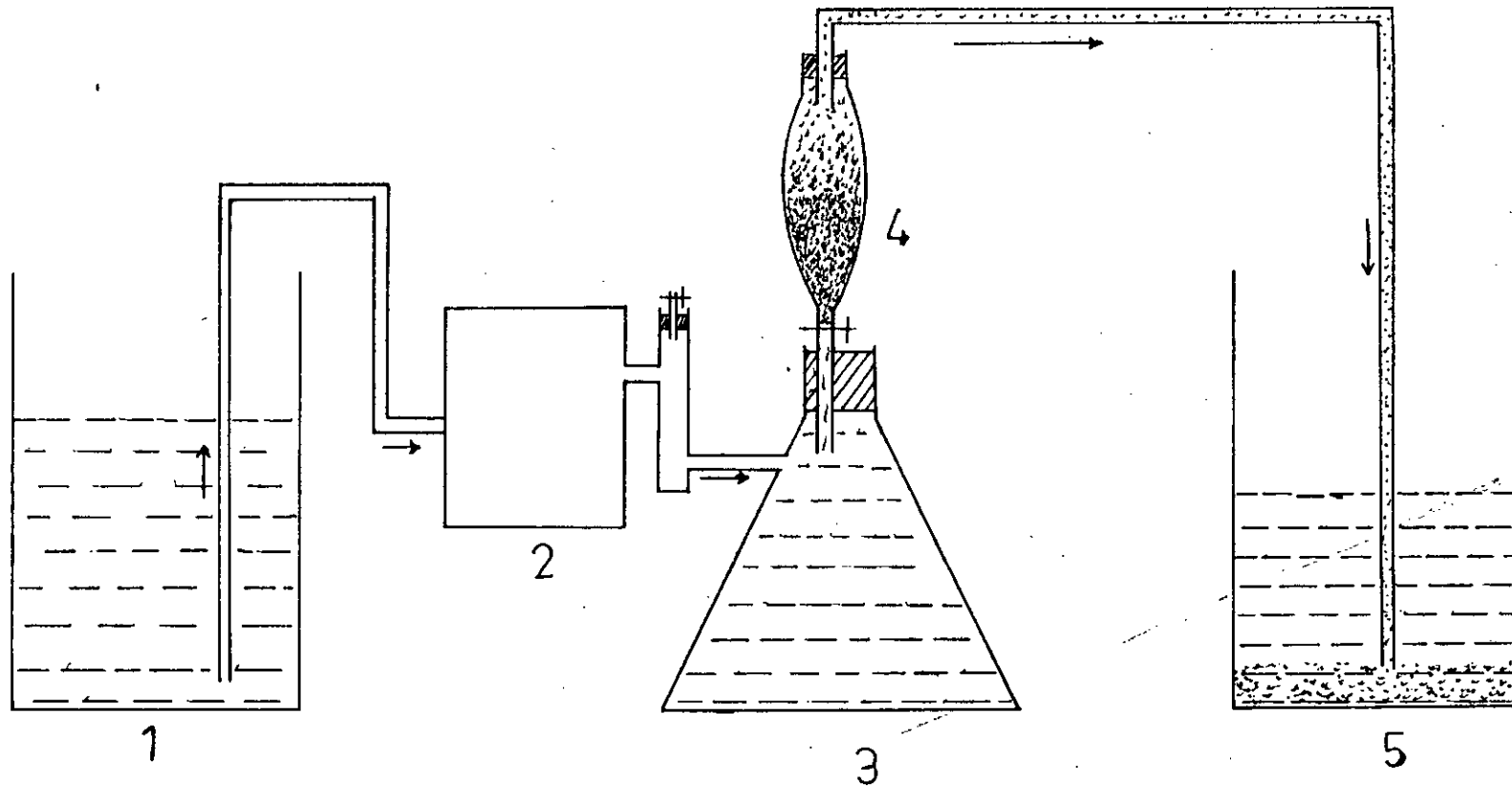


1. Water Reservoir
2. Pump
3. Bottom Reservoir for slurry of particles, feeds the separating funnel
4. Separating funnel
5. Collecting basket

## TABLE 5-1

### Physical Properties of HPLC Packing Particles and Suppliers

| Material | Description. | Particle Size | Approx. Surface Area ($S_{BET}$ $m^2gr^{-1}$) | Manufacturers |
|---|---|---|---|---|
| Spherisorb A20Y | Micro-particulate Spherical porous alumina | - 20 $\mu$m | 93 | Phase Separations Ltd., Deeside Industrial Estate, Queensferry, CLWYD. |
| Spherisorb A10Y | " " | 10 $\mu$m | 93 | " " |
| Hypersil | Micro-particulate Spherical porous Silica gel | 5 $\mu$m | 200 | Shandon, London, G.B. |
| Hypersil ODS-TMS | Bonded Phase Micro-particulate Sperical porous Silica gel | 5 $\mu$m | 200 | " |
| Zirconium oxide gel | Micro-particulate Sperical Porous Zirconia (S grade) | 10 $\pm$ 3 $\mu$m | 9 | Magnesium Elektron Manchester, G.B. |

The names, characteristics and addresses of suppliers of the packing

materials used in this study are listed in Table 5-1.

### 5.3.4 Experimental Techniques

Zirconium oxide gel was kindly supplied by Magnesium Elektron Ltd.

and was their highest purity grade, with the spherical particles sized

between 3 and 23 $\mu$m. Particles of a narrow diameter range ( 10 $\pm$ 3 $\mu$m)

were isolated by hydraulic elutriation (100). The fractionation apparatus

is illustrated by Figure 5-4. A slurry in water of the original mixture of

particle sizes (3-23 $\mu$m) was placed in a separating funnel (1 L.) and in

the reservoir beneath and pumped upward through the funnel to a collection

vessel. Elutriation with water at 150 $cm^3$ min. separated very small and

medium size particles from very large particles (a total of 40 L.) Then

the fraction carried over at 150 $cm^3$ min.$^{-1}$ was replaced in the separating

funnel and elutriated at 70 $cm^3$ min.$^{-1}$. The very small particles were

separated from the residual dp $\simeq$ 10 $\mu$m particles suspended in the funnel.

Particle diameter was measured with a travelling stage on a microscope.

The zirconium oxide particles (dp $\simeq$ 10 $\mu$m) were slurry packed after

fractionation and then tested as a chromatographic adsorbent using hexane

and methanol (99:1) as solvent and para aminoazobenzene and aminoazobenzene

as model solutes but there was no measurable adsorption of these compounds.

Activation was apparently needed and this was done through hydrothermal

treatment. The zirconia gel was suspended in water and heated in a

sealed tube at 150$^o$C for 5 hours. If zirconia behaves in an analogous

fashion to silica, pore diameters should have been increased and surface

area should have been reduced by the above process (cf. De Baer and Vleeskens,

95) and the polarity of the surface should also have been increased.

The modified zirconia particles were used in the experiments described below.

Sulphonic acid dyestuffs and aromatic hydrocarbons were used as model solutes and are shown in Appendix 1.

The purity of the methanol solvent used in these experiments proved to be critical for reproducible measurements. Attempts to use "Reagent Grade" methanol from several suppliers gave variation in solute retention over a four-fold range. Consistent solvent action was obtained from A.R. grade methanol only after distillation from magnesium methoxide solution. HPLC grade methanol obtained from Rathburn Chemicals gave retention values essentially identical to those with purified A.R. grade. Double deionised water was used to prepare mixtures, which were degassed by boiling under reflux before use.

Hexadecyltrimethylammonium bromide, puriss (CTAB) was purchased from Fluka (Buchs, Switzerland) and was used as surface active pairing ion agent in these experiments.

## 5.4    Column Testing and Calculation of Results

Columns were tested after packing to avoid poor experimental chromatographic measurements.

If it is assumed that a retained solute is fully resolved then the distribution profile of the eluted zone will be approximately Gaussian in shape. Hypothetical solute retention was shown in Figure 2-1.

The number of plates, N, from this Gaussian profile can be calculated as:

$$N = 16 \left(\frac{t_r}{W_t}\right)^2 = 5.54 \left(\frac{t_r}{W_{\frac{1}{2}}}\right)^2 \qquad 5\text{-}10$$

Where $t_r$ and $W_t$ are the retention time and base (time) width of the retained solute zone respectively, and $W_{\frac{1}{2}}$ is the half height (time) width of the zone.

Plate height can also be expressed as:

$$H = \frac{L}{N} = \frac{L}{16} (\frac{W_t}{t_r})^2 = \frac{L}{5.54} (\frac{W_{\frac{1}{2}}}{t_r})^2 \qquad \text{5-11}$$

Where L is the column length.

Reduced plate height is defined as:

$$h = \frac{H}{dp} = \frac{L}{16\ dp} (\frac{W_t}{t_r})^2 = \frac{L}{5.54\ dp} (\frac{W_{\frac{1}{2}}}{t_r})^2 \qquad \text{5-12}$$

Where dp is the mean column packing particle diameter. For accurate calculation of reduced plate height it is necessary to use an accurate value of the mean particle diameter, but when microparticles with diameter less than 40 $\mu$m are used, sieving procedures for assessment of particle size do not work. Experimentally, the mean value of one hundred microscopic measurements are required. It is estimated that $\pm$ 10% error in microscopic measurements of very small particles (less than 10 $\mu$m in diameter) is inevitable. Indirect estimation of particle diameter may be accomplished using the column resistance parameter, in which effective particle diameter may be calculated from column packing permeability. It is given as:

$$\varphi = \frac{\Delta p.dp^2.tm}{\eta\ L^2} \qquad \text{5-13}$$

Where $\Delta p$ is the operating pressure drop and $\eta$ is eluent viscosity. Well-packed columns give values of $\varphi$ between 500-1000 (500 for good slurry packing). The equation above shows that $\varphi$ and dp are dependent on each

other, thus it is possible to calculate one variable from measurements of the other. In other words, if large particles are used, dp can accurately be measured and so $\varphi$ may be calculated from equation 5-13. On the other hand if the particles are very small, then a value may be assumed for $\varphi$ and then the effective particle diameter calculated.

Reduced velocity $\upsilon$ can be expressed as:

$$\upsilon = \frac{U.dp}{D_m} \qquad\qquad 5\text{-}14$$

Where U is the linear velocity of an unretained and non-excluded eluite ( $U = \frac{L}{t_m}$ ) and $D_m$ is the diffusion coefficient of an eluate molecule. Some viscosities and typical values of $D_m$ for some popular solvents are given in Table 5-2 in which it is assumed that the optimum linear velocity U corresponding to $\upsilon = 7$ for dp = 5 $\mu$m.

If the reduced plate height, h, is plotted versus reduced velocity, an efficient column should show h values between 2-10 at $\upsilon$ between 3 and 7.

Phase capacity ratio can be calculated from the following expression:

$$K' = \frac{t_r - t_m}{t_m} \qquad\qquad 5\text{-}15$$

Where $t_r$ and $t_m$ are the retention times of a retained solute and an unretained solute, which is shown schematically in Figure 2-1.

TABLE 5-2

Viscosities ( $\eta$ ), Diffusion Coefficient (Dm) and Optimum Linear Velocities

| Eluent | $\eta$ (NSm$^{-2}$) | $D_m$ ( m$^2$ s$^{-1}$) | $U_{opt}$ (mm.s$^{-1}$) |
|---|---|---|---|
| Hexane | $0.33 \times 10^{-3}$ | $4 \times 10^{-9}$ | 4 |
| Water | $1.0 \times 10^{-3}$ | $1 \times 10^{-9}$ | 1 |
| Methanol:Water (40:60) | $1.8 \times 10^{-3}$ | $0.6 \times 10^{-9}$ | 0.6 |
| Methanol | $0.6 \times 10^{-3}$ | $1.9 \times 10^{-9}$ | 1.5 |

FIGURE 5-5

Relationship of logarithm of reduced plate height
(log h) to logarithm of reduced velocity.
Eluent: Hexane : Methanol (99:1 V/$_V$).
Column packing: Zirconium Oxide. Eluite: Aminoazobenzene.
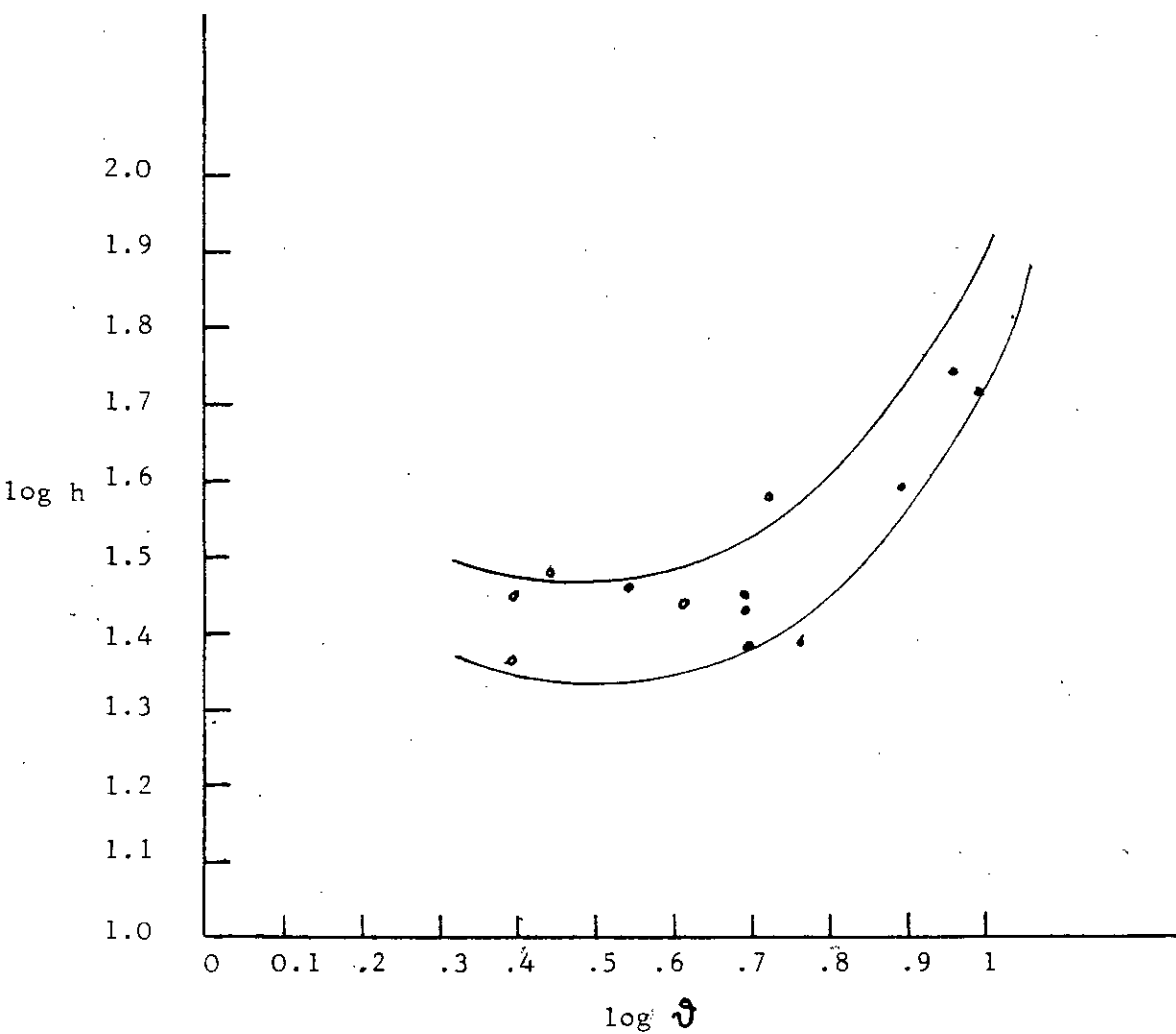
## 5.5    Results and Discussion

In the initial experiments, two columns were packed; one with Hypersil and another with zirconium oxide. Column efficiency was measured on the columns operated in an adsorption mode using hexane: methanol (99:1) eluting solvent and paraaminoazobenzene and aminoazobenzene ($\lambda$ max was obtained from UV spectrum), as model eluites. Columns of 125 mm length and 4.6 mm bore gave satisfactory results for Hypersil (N $\simeq$ 7000-8000 plates), but as is shown in Figure 5-5, the reduced plate height for zirconia was 25-30. The reason could be the low surface area of zirconium oxide which may indicate that the structure of the pores in this zirconia was not really suitable for HPLC usage.

After efficiency testing both columns were switched to "reversed phase" elution conditions and subsequently further experiments were attempted on the same silica column.

## 5.6    Separation on Silica Gel

An equal volume (1:1) mixture of methanol and water was used as eluent in all measurements quoted in Figures 5-6 and 5-7 to obtain reasonable retentions for the sulphonic acid and aromatic hydrocarbon sample substances. Although the order of retention for a given group of solutes does not change with eluent composition, capacity ratios of the different solutes are dependent on solvent composition. The effect of changes of eluent composition will be discussed later in this Chapter.

Complete equilibration of the column packing with solvent-surfactant is very critical for consistent retention behaviour, which depends upon several factors. Column equilibration time varies with

different packing materials (e.g. silica, zirconia, etc.) and it also

depends upon the kind and concentration of surfactant dissolved in the

eluent. The longer the surfactant chain and also the higher is its

concentration, then the shorter is the time required to reach

equilibrium. Complete equilibrium can be tested by consecutive

injections of the same model eluite (S). Constant retention of solute

demonstrates that the surface equilibrium is complete. Using CTAB as a

surfactant gave 0.5-1 hour equilibrium times for silica and ca 15 min.

for zirconia at a flow rate of 1 $cm^3 min^{-1}$.

All data used to establish the relationship between log K' and

CTAB concentration were collected from columns which had previously

been exposed only to solvent containing the measured or lower concentrations

of the same added surfactant. This precaution was thought to be necessary

since it was observed that attempts to convert a silica-CTAB column to

one in equilibrium with a different quaternary ammonium salt

(benzyltrimethylammonium chloride and propyltrimethylammonium iodide

were used in preliminary experiments) gave retention data different from

those of silica columns which had been in contact with these other salts

only. Moreover, several attempts to regenerate a "clean" silica surface

by acid washing (0.1 $mol.dm^{-3}$ acetic, formic and nitric acids were

examined) to remove "bound" surfactant did not generate a surface

capable of interacting to the original extent with added CTAB. Although

these "acid washed" columns did not retain their surfactant binding

properties, the washing process did apparently give a silica surface

similar to the original "as packed" condition, since measurements of

FIGURE 5-6

Relationship of sample retention (K') to concentration
of added surfactant (CTAB). Eluent methanol:water
(50:50 $V/_V$).

Column packing: Hypersil. Flow rate 1 $cm^3.min^{-1}$.
Eluites coding as in Appendix 1.
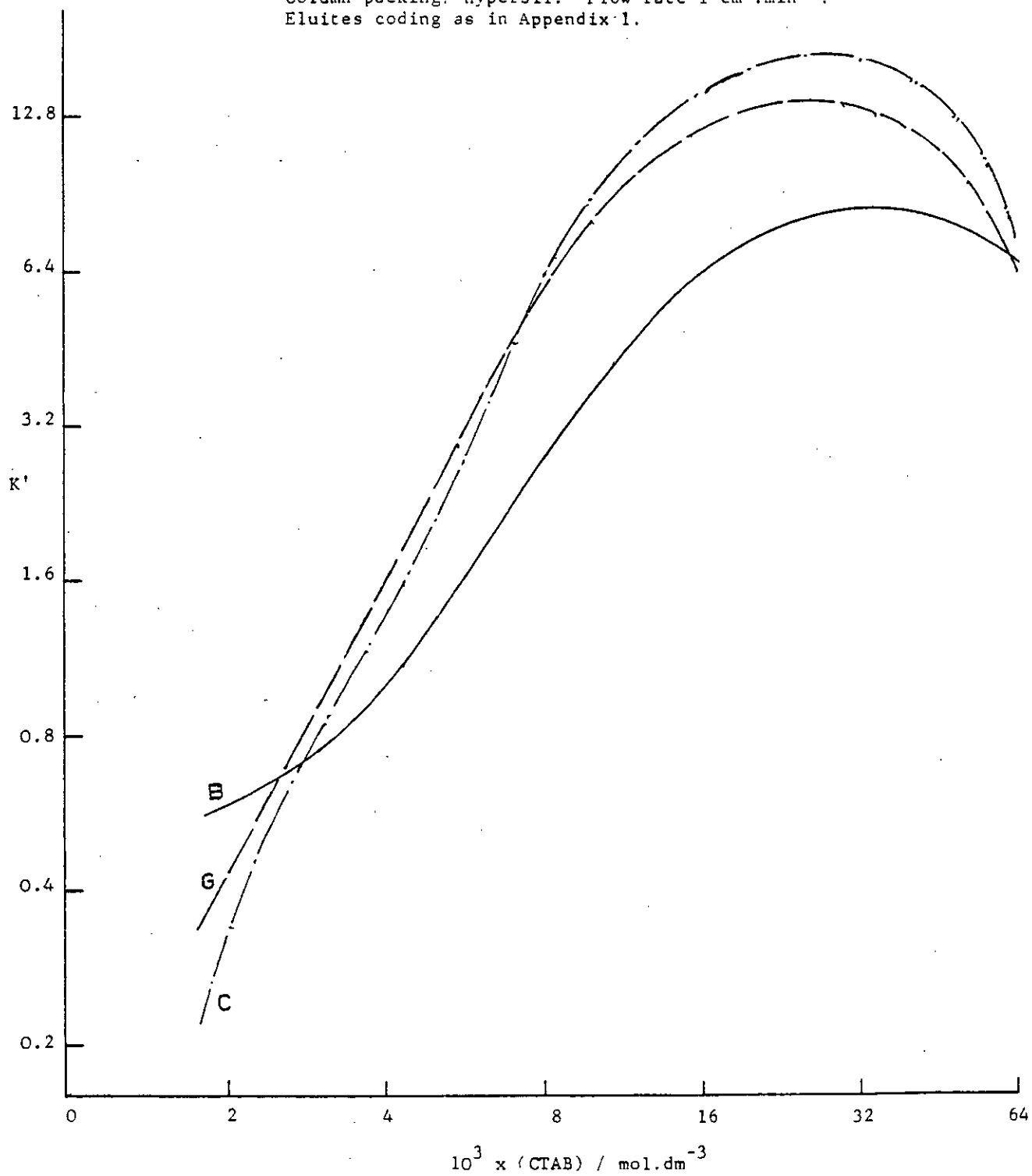
retention of some standard solutes in the adsorption liquid chromatographic mode with 1% methanol in hexane as solvent were essentially identical before and after surfactant-acid treatment. In spite of this clear evidence of a surface which could not be readily regenerated on the column packing, it was found that retention data from several columns which were equilibrated in the same way with the same surfactant were consistent. Furthermore, the relative characteristics of fully equilibrated CTAB-silica columns were constant over periods of several weeks.

### 5.6.1  The Effect of CTAB Concentration on Sulphonic Acid Retention on Silica Gel

The effects of variation of the CTAB concentration (in 1:1 methanol:water) on the retention of three sulphonic acids is shown in Figure 5-6. The mechanism of retention as explained thoroughly in Section 5-2, is apparently due primarily to the hydrophobic interaction between the dynamically hydrophobised silica surface and ion pairs formed between the sulphonic acids and CTAB present in the mobile phase or could equally well be an ion exchange process for the sulphonic acids. CTAB concentrations vary from $1.7 \times 10^{-3}$ to $5.5 \times 10^{-2}$ mol.dm$^{-3}$ in the solvent mixtures. When no CTAB was present in the mobile phase there was no retention for any of the analytes. It may be seen that as the concentration of cetrimide increases from $1.7 \times 10^{-3}$ to $20 \times 10^{-3}$ mol.dm$^{-3}$, the retention of the different solutes also increases. Although the shapes of the retention versus (CTAB) curves are similar, several changes in the order of elution of solutes are evident from the crossover points

of the various curves. Differences in selectivity of different analytes may arise from differences in the chemical reactivities of these analytes, and this may be an advantage in chromatography in that it may often be possible to control the relative solute retention by alteration of the surfactant concentration.

In the paper on soap chromatography by Knox and Laird (36), maximum retention of sulphonic acid analytes was found at ca $7 \times 10^{-3}$ mol.dm$^{-3}$ (CTAB), but in this present work maximum retention was observed at higher CTAB concentrations (ca $2.7 \times 10^{-2}$ mol.dm$^{-3}$). This observation may be partially explained by the lower surface area of the particles (200 m$^2$.g$^{-1}$) used in this work compared with that used by Knox and Laird (500 m$^2$.g$^{-1}$). On the other hand, Horvath et al (53) suggested that the shape of these curves is a function of a balance of interaction between the packing surface and the solvent, and therefore the two sets of data should differ, since the methanol:water (1:1) eluent of this investigation is considerably less hydrophobic than the water:propanol (1:3) eluent of the earlier studies. The difference can be seen in the lesser retention of Schaffer's acid ($K' \simeq 0.15$) when 1:3 ($^V/v$) water:propanol was used as a solvent, compared with a K' value about 13 when 1:1 ($^V/v$) water:methanol solvent was used.

The amount of cetrimide adsorbed on the silica surface was measured by the colorimetric method outlined by Knox and Laird (36) and Tomlinson, et al (86), in which consecutive 0.2 ml. aliquots of eluent were added to sample tubes containing equal values of propanol, methylene chloride and water (2 mls. of each) containing Sunset Yellow. When this

mixture was mixed by shaking and allowed to separate into two layers, the dye was strongly partitioned into the aqueous layer. However, when the eluent contained CTAB, dye was extracted into the organic phase as the ion-pair. The amount of ion pair can be calculated from photometric measurement of the concentration of the dyestuff anion in the separated organic layer. When the extinction coefficient of the organic solution of the dye ion-pair was plotted against the time of elution, a diagram which resembled that illustrated below resulted. The amount of adsorbed CTAB, $q$, was calculated from this diagram as:

$$q = (c_2 - c_1) \cdot Fc \cdot (t_1 - t_0) \left(\frac{W_A + W_B}{W_A}\right) \qquad \text{5-16}$$
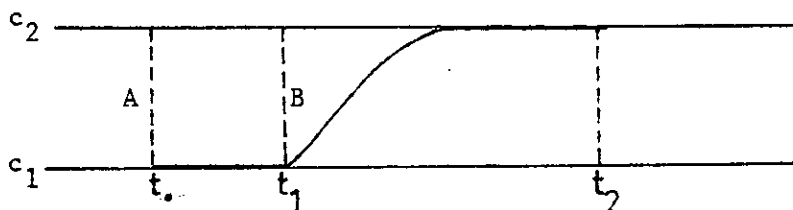


Where $c_2$ is the concentration at equilibrium, Fc is the flow rate, $t_0$ is the retention time of unretained solute and $W_A$ and $W_B$ refer to the weight of chart paper of areas A and B.

At $1.4 \times 10^{-2}$ mol.dm$^{-3}$ (CTAB) it was found that 64 mg of surfactant were sorbed on 1 g of silica, which infers an area coverage of $0.8 \times 10^{-6}$ mol.m$^{-2}$ of silica surface, roughly equivalent to one sixth of the maximum possible coverage if sorption proceeds by $-\overset{+}{N}(CH_3)_3$ reaction with the surface. The greatest reported silica surface coverage by covalently attached $- Si(CH_3)_3$ groups is ca $4 \times 10^{-6}$ mol.m$^{-2}$ (96).

FIGURE 5-7

Relationship of sample retention (K') to concentration
of added surfactant. Eluites: Coding as in Appendix 1.
Column and conditions as in Figure 5-6.



K'

12.8

6.4

3.2

1.6

0.8

0.4

0.2

F

A

D

E

1    2    4    8    16    32    64

$10^3$ x (CTAB)/mol.dm$^{-3}$
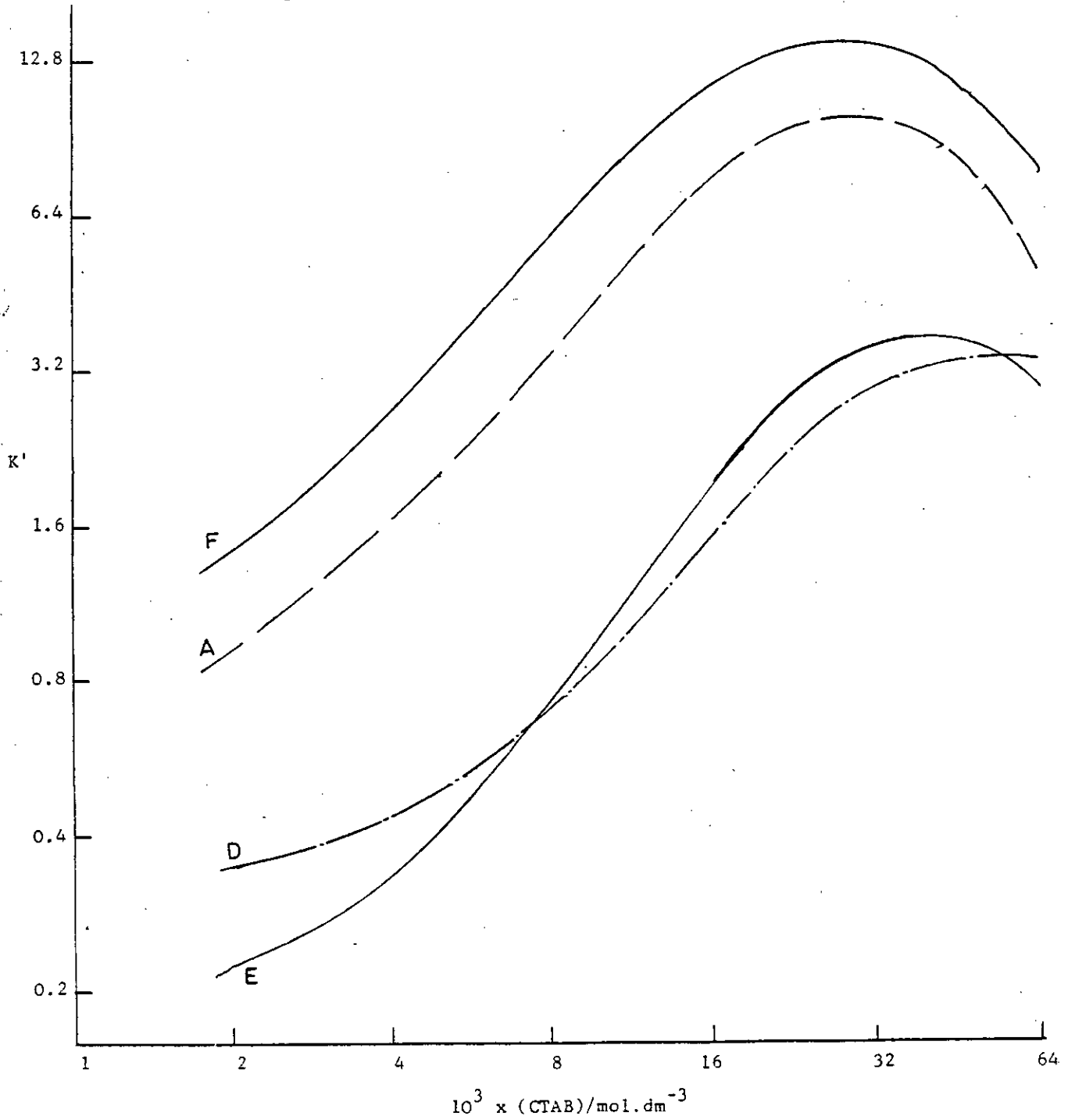
## 5.6.2  Separation of Non-Polar Aromatic Hydrocarbons on Silica Gel

Non polar analytes were also successfully separated on silica packings by elution with aqueous methanol containing small amounts of CTAB. There was no retention in the absence of the surfactant. There is apparently no interaction between the hydrophilic silica surface and lipophilic aromatic hydrocarbons, but retention will occur on this surface as it becomes equilibrated with CTAB. The relationship between K' and (CTAB) for some aromatic hydrocarbons is shown in Figure 5-7.

The differences between capacity ratio and selectivity ( $\alpha_{K'_1,K'_2} = \dfrac{K'_2}{K'_1}$ ) of aromatic hydrocarbons on solvent-generated hydrophobic packings and an ODS-TMS bonded-phase packing are shown in Table 5-3. It is obvious from this Table that in spite of large differences in absolute retentions of the analytes, both the order of elution and selectivity of separations on the three packings demonstrate the apparent similarity in the chromatographic characteristics of these three different column packings.

Another variable which may lead to an increase in capacity ratio is the surfactant chain length. The longer the alkyl chain, the greater should be the retention of aromatic hydrocarbons, but lack of suitable samples of alkylmethylammonium halide prevented a detailed investigation of this effect. Preliminary investigations with propyltrimethylammonium ( $CH_3-CH_2-\overset{+}{N}(CH_3)_3$ ) and benzyltrimethylammonium ( $C_6H_5CH_2-\overset{+}{N}(CH_3)_3$ ) halides showed that capacity ratio does indeed increase with chain carbon number. The effect of chain length in reversed phase chromatography on alkyl bonded silica was the subject of a number of investigations by Hemetsberger,

FIGURE 5-9

FIGURE 5-8

Separation of four non-polar aromatic compounds (indicated in Figure 5-8) on a column 125 x 5 mm of ODS Hypersil. Eluent : methanol : water $(60:40, V/_V)$. Conditions as in Figure 5-6.
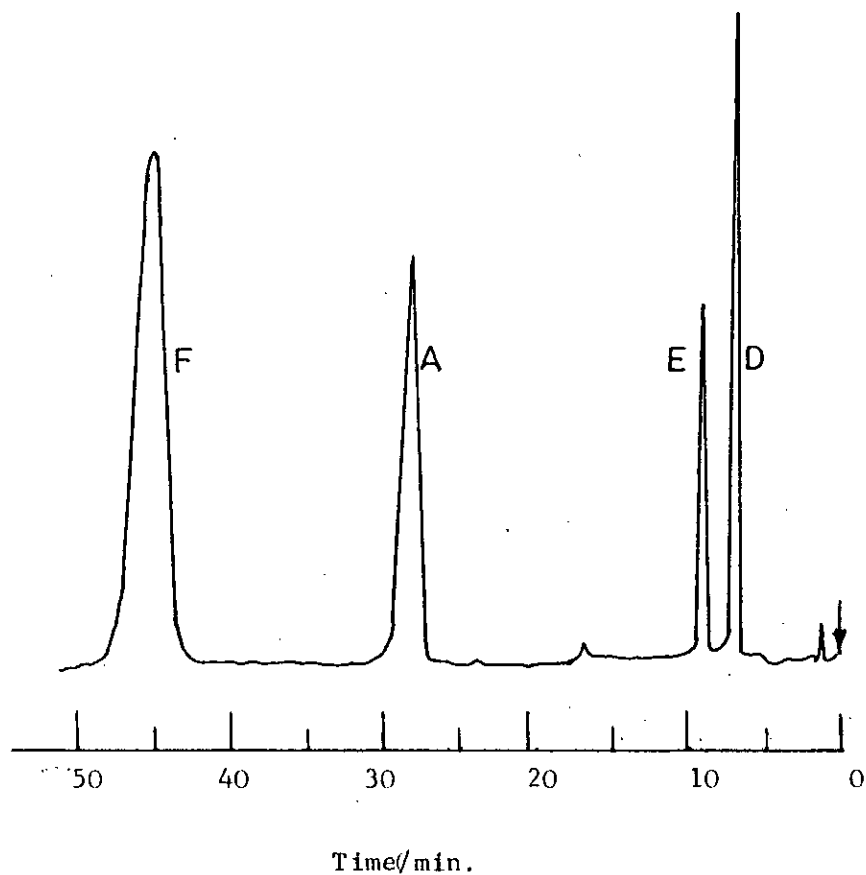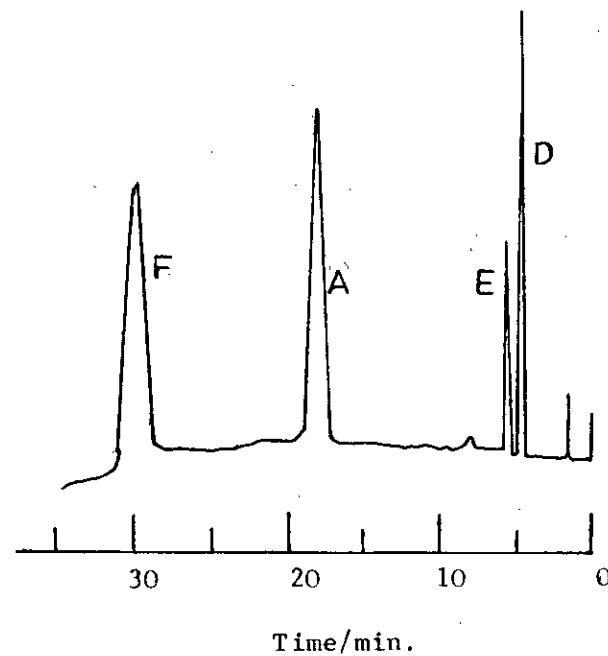
Separation of non-polar aromatic compounds (coding as in Appendix 1). Column and conditions as in Figure 5-6.



Time/min.



Time/min.

et al (97-99). They found that retention increases with increasing

percentage carbon bonded to the adsorbent. Similarly, Tomlinson, et al

(86) discussed the increase in solute retention with increasing surfactant

chain length in cationic "soap" chromatography on octadecyl silica. If

the retention mechanism in these investigations is as closely related

to that of the alkyl bonded phases as the preceeding results suggest,

then a similar dependence of K' upon chain length of the added surfactant

should be predicted for the solvent-generated system.

TABLE  5-3

Comparative Retention Data ($1.4 \times 10^{-2}$ mol.dm$^{-3}$ CTAB)

| Analyte | Hypersil | ODS-Hypersil | Zirconia |
|---|---|---|---|
| $K'_1$ Fluorenone | 2.3 | 3.7 | 0.38 |
| $K'_2$ Naphthalene | 2.7 | 5.0 | 0.63 |
| $K'_3$ Anthracene | 11 | 17 | 2.5 |
| $K'_4$ Pyrene | 19 | 28 | 4.1 |
| $\alpha_{1-2} = \dfrac{K'_2}{K'_1}$ | 1.1 | 1.4 | 1.7 |
| $\alpha_{2-3} = \dfrac{K'_3}{K'_2}$ | 4.0 | 3.5 | 4.0 |
| $\alpha_{3-4} = \dfrac{K'_4}{K'_3}$ | 1.7 | 1.6 | 1.7 |

Figure 5-8 is a representative chromatogram of simple mixtures of neutral

aromatic analytes using methanol/water/CTAB solvent systems with a

spherical silica column packing, and Figure 5-9 is a chromatogram of the

FIGURE 5-10

Relationship of sample retention (K') to
concentration of cetrimide
Eluent, eluite and conditions as
Figures 5-6 and 5-7.  Column packing:
hydrothermally modified zirconia ($dp \cong 10$ $\mu$m)
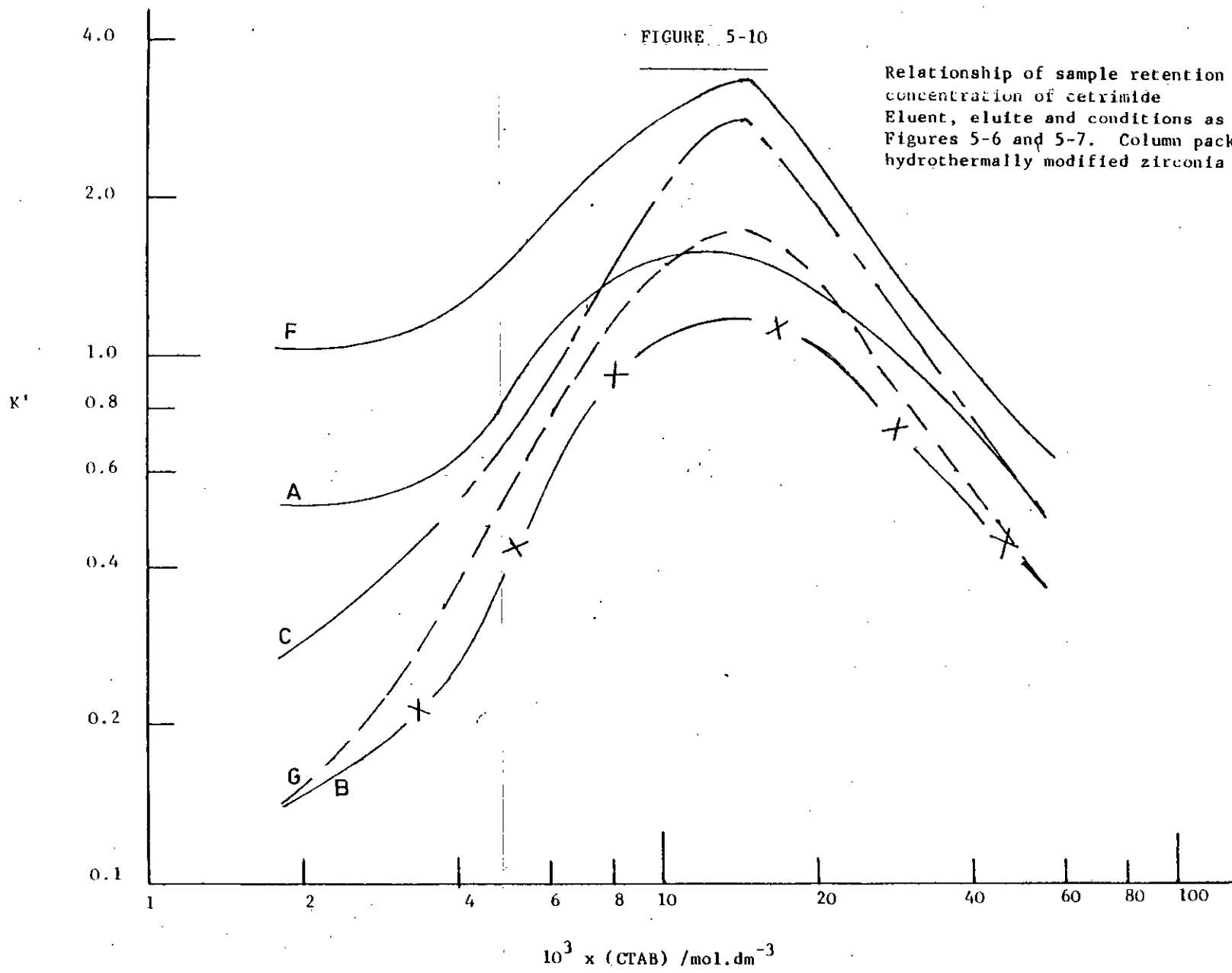
same mixture with the same eluent used in Figure 5-9, but on the octadecyl "bonded phase" ODS Hypersil. As suggested by the data of Table 5-3, the two separations are very similar.

## 5.7 Separation of Sulphonic Acids and Non-Polar Aromatic Hydrocarbons on Zirconia Packing

Porous zirconia gel ($ZrO_2$) is an acidic oxide which is chemically similar to silica. Appendix 2 shows the physical properties of zirconium oxide "S" grade which was used in our experiments.

A potential advantage of zirconia was its apparent stability over a wide range of pH, and particularly in strongly basic aqueous solutions where silicas and aluminas slowly dissolve (pH up to 11 and 12). A stability test was carried out with approximately 1.5 gr. of zirconia particles using 50 $cm^3$ of standard buffer solutions of pH 8-12. The $ZrO_2$ particles remained in contact with the buffers for a week during which they were vigorously shaken twice daily. At the end of the week the particles were filtered, washed, dried, and weighed. There was no significant loss in weight of the zirconia particles, even in those kept in aqueous solution at pH 11 and 12. This experiment was repeated several times and no weight losses were recorded.

Investigation on zirconia columns was carried out with the same eluent systems as in the previous work on silica. Figure 5-10 shows the relationship between K' and (CTAB) on columns packed with zirconia for model analytes. Lesser retentive power would be predicted for zirconia ($9 m^2.gr^{-1}$ calculated pore = 36 nm) than for the silica ($200 m^2.gr^{-1}$

FIGURE 5-11

Separation of four non-polar aromatic compounds
as in Figure 5-8. Column packing as in Figure 5-10.
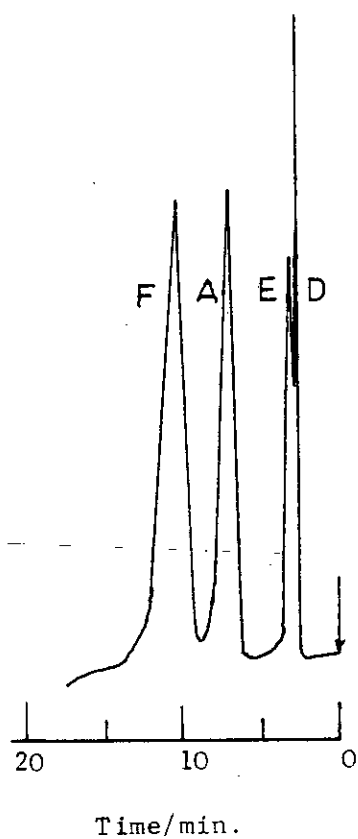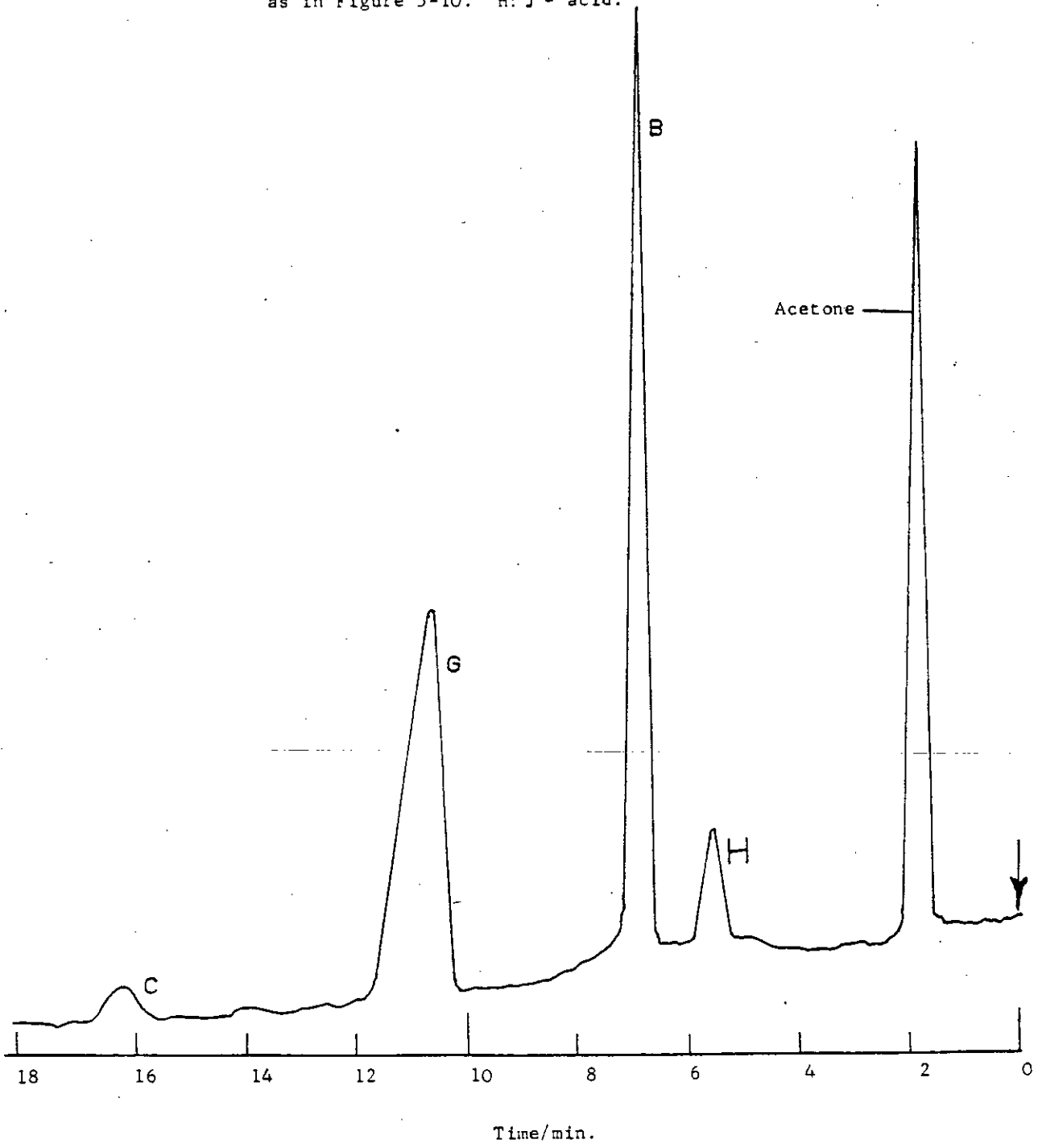Flow rate 0.6 $cm^3.min^{-1}$.



Time/min.

FIGURE  5-12

Separation of four sulphonic acids as (CTAB)
ion pairs on the zirconia column of the Figure
5-11.  Eluent, eluites and operating conditions
as in Figure 5-10.  H: J - acid.



Time/min.

calculated pore = 12 nm), but the disparity in "available" surface is not as great as it appears, since the packing surface area in a typical 125 mm long column would be about 240 $m^2$ for the silica (bulk density ca 0.5 gr.cm$^{-3}$) and 43 $m^2$ for the zirconia (bulk density ca 4 gr.cm$^{-3}$). In fact K' at 1.4 x 10$^{-2}$ mol.dm$^{-3}$ (CTAB) for pyrene was ca 19 on silica and 4 on zirconia, so retention is slightly higher on the latter packing than a simple surface area ratio would suggest.

Plate height curve (Figure 5-5) and the chromatograms shown in Figures 5-11 and 5-12 show that column efficiency is very low when zirconium oxide was used as HPLC packing, even after careful size fractionation and thermal treatment. At the time of writing there has not been significant research on zirconium oxide surface characteristics, and especially on surface treatment, to allow final judgement on its suitability for HPLC use. The importance of the resistance to attack by aqueous alkali of this material emphasises the need for more detailed study.

The polarity of zirconia adsorption sites can be compared with that of the silica surface by examination of Figures 5-6, 5-7 and 5-10. These show that maximum analyte retention is attained at 2.7 x 10$^{-2}$ mol.dm$^{-3}$ (CTAB) on silica, while maximum retaining power for zirconium oxide lies at 1.4 x 10$^{-2}$ mol.dm$^{-3}$.

Investigation of the ceria surface by Gilbert and Wall (93) showed that the equivalent maximum for ceria lies between 3 x 10$^{-3}$ mol.dm$^{-3}$ and 7 x 10$^{-3}$ mol.dm$^{-3}$, which places it as lowest of these three oxides. The order of surface polarity may accordingly be written as silica$>$zirconia$>$ceria.

FIGURE   5-13

Relationship of sample retention (K') to volume% methanol
in eluent at constant (CTAB) (7 x 10 mol.dm$^{-3}$).   Column
packing as in Figure 5-10.   Analytes coding as in Appendix 1
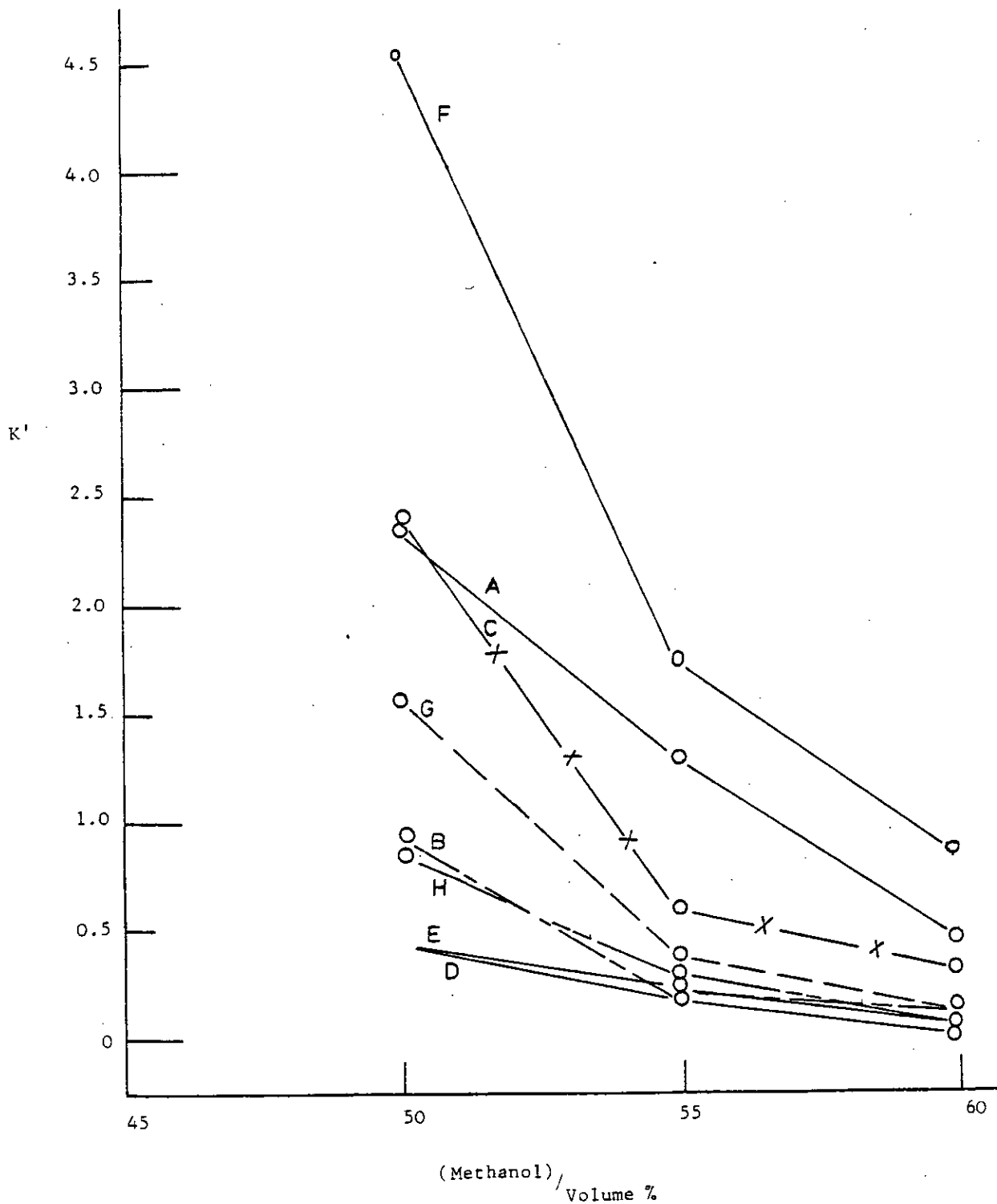
This comparison is not fully justified since the solvent composition used in the measurements on silica and zirconia is different from that used with ceria. Experiments to establish the maximum retention on zirconia obtained with the same solvent used in the earlier work on ceria showed that maximum retention was indeed at $1.4 \times 10^{-2}$ mol.dm$^{-3}$ (CTAB).

5.8    Effect of Organic Modifier Concentration on Solute Retention

Solute retention can also be controlled by variation of the concentration of organic modifier in the eluting solvent system. In the introduction it was pointed out that retention should decrease with any increase in the proportion of organic modifier in the solvent system. This effect was observed in our investigations. When the concentration of methanol was increased in the eluting solvent, analyte capacity ratios were reduced, as is evident from Figure 5-13 in which variation of K' with percentage (by volume) of methanol eluent (containing $0.7 \times 10^{-2}$ mol.dm$^{-3}$ CTAB) is plotted for a range of solutes. Selectivity of separation was also affected by organic modifier changes although this latter effect is rather less pronounced than solvent-induced changes in retention.

5.9    Conclusions

Data from this study and that from previous investigations (36, 93) on the interaction between cationic surfactant dissolved in the eluent phase and LC column packings prepared from porous gels of cerium (IV) oxide, silicon (IV) oxide or zirconium (IV) oxide may be interpreted as arising from the "in situ" generation of a strongly

(probably electrostatically) bonded alkyl hydrocarbon surface phase. Horvath, et al (Ref. 53 Figure 12) have shown that the relationship between concentration of surfactant and K' for a given analyte is a function of both the alkyl chain length of the surfactant (i.e. hydrophobicity) and the polarity (i.e. water content) of the eluent. So, as the hydrophobic character of the stationary phase is increased with a constant composition solvent, a corresponding decrease in the surfactant concentration at maximum solute retention would be predicted. Such a decrease is observed with the silica, zirconia and ceria stationary phases used in this and the preceding studies (93). When this observation is combined with the fact that the relative retention of hydrocarbon solutes in the CTAB silica system is very similar to that observed on octadecyl silica, it seems clear that an eluent-generated hydrophobic surface is the main retentive agent in the surfactant-oxide systems.

Both absolute and relative retention of polar and non-polar analytes are shown to be controlled by solvent composition both with respect to the bulk organic components (i.e. methanol, propanol, etc.) and the trace surfactant. Additional control of the parameters affecting a given separation may be obtained by use of oxide gels with differing surface characteristics, as in classical adsorption chromatographic practice. This extension to a wider range of analytes (and a novel zirconia column packing) of the "soap chromatographic systems described in a study by Knox and Laird (36) of separations of polar sulphonic acids as ion pairs on an alkyl bonded silica and on silica column packings would appear to be a useful additional to the LC separation modes in current use.

CHAPTER   6

Anionic Surfactant Effects on Alumina

6.1     Introduction

A major new technique for separation of amines arose from
the use of n-alkyl chemically bonded phases as column packing
materials for reversed-phase high performance liquid chromatography.
Two distinct modes of separation of amines and amino acids on these
materials have been reported.  Firstly, the more conventional form
used aqueous buffer with addition of a suitable water-miscible
organic solvent.  The retention mechanism in this so called "solvophobic
chromatography" was investigated intensively by Horvath et al (52).
In the other mode, commonly referred to as RP-IPC, the retention of
ionized compounds is strongly enhanced by addition of suitable
lipophilic ions to the aqueous mobile phase.  A comprehensive review
of ion pair chromatography by Tomlinson, et al (61) clearly
demonstrated that the separation of amines, amino acids and peptides
using RP-IPC system is a practical and very rapid alternative to
conventional techniques of analysis or purification, e.g. thin layer
or ion exchange chromatographic techniques.

In a paper on "Solvent Generated Hydrophobic Chromatography"
Wall (102) explained that since the porous silica surface is acidic
separation of peptides and amino acids can be achieved on this oxide
surface by means of double anionic-non ionic interaction between the
surface and surfactants.  The mechanism of separation as explained by

this author was more likely ion exchange between counter-cation of the surfactant and the cationic end of the amino acid.

In our previous study (101) (Chapter 5) it was shown that anionic species as well as aromatic hydrocarbons and ketones can be successfully separated on hydrophilic silica gel after dynamic interaction between the cationic end of the surfactant and the acidic silica surface has occurred.

Accordingly this present study describes the effects of addition of anionic surfactants to aqueous methanolic eluents of columns packed with aluminium III oxide gel particles. These commercially available $\gamma$ - alumina packings are known to possess basic as well as acidic adsorption sites, and may be shown to adsorb and retain small amounts of anionic surfactants from external aqueous methanolic solutions.

Furthermore, as has been previously demonstrated with cationic (101) surfactants and non-ionic detergents (112) (Chapter 7), retention of both charged and uncharged eluites increases with increasing alkyl carbon chain length of anionic detergent. However, Armstrong and Terrill (103) showed that in aqueous "micellar" solution of high surfactant concentration, non polar analytes tend to be less strongly retained on the alumina/surfactant interface than charged analytes.

## 6.2    Experimental

Complete description of the porous alumina used in the majority of these experiments was shown in Table 5-1. Some confirmatory studies

were also carried out on Lichrosorb Alox T (E. Merck A.G. Darmstadt, Germany, dp = 10$\mu$m, $S_{BET}$ = 70 m$^2$.gr$^{-1}$) in our experiments. Alumina from different suppliers showed that for reproducible results, it is important to calcinate these particles in air (24 hours at 600$^o$C). Packing techniques have been described in Chapter 5. In order to prevent the effects of different purification procedures of the organic modifier (methanol) in our results, special HPLC grade methanol (Rathburn Chemical Limited, Walkerburn, Scotland) was used in all procedures. Sodium Lauryl Sulphate (SDS), "Puriss", and Tergitol 7 (T.7) sodium "heptadecyl" sulphate were purchased from Fluka A.G., Buchs, Switzerland. All other solvents and reagents used were of reagent grade and were used as received from the suppliers. Water was distilled from glass. The column temperature was controlled with column jackets heated by a thermostat to ca 25$^o$C. Bulk solvents were degassed by refluxing and the appropriate mobile phases were prepared and equilibrated to operating conditions as reported previously (101). All columns were equilibrated with new mobile phase systems for at least one hour.

6.3     Results and Discussions

6.3.1     Sodium Lauryl Sulphate (SDS) Effects

A solution of (1:1) $^V$/v methanol/water was used as mobile phase base in all the present experiments. Different concentrations of SDS was prepared in the aqueous methanol base and applied to alumina oxide gel column. The first observations with very low concentrations of surfactant showed that very large volumes of eluent

FIGURE 6-1

Variation of retention with change in SDS concentration.

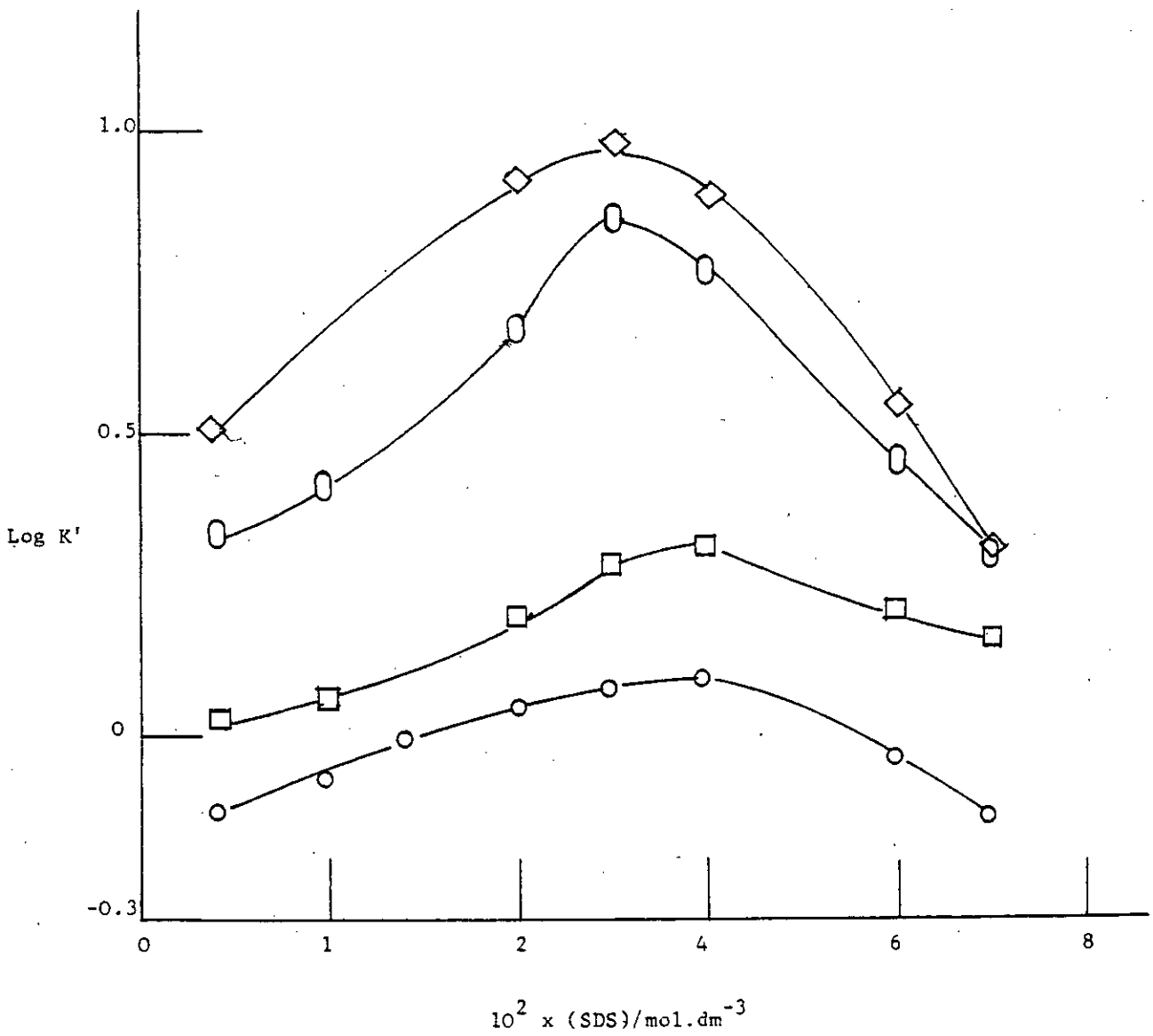○ - 9-fluorenone;  □ - naphthalene;

◖ -anthracene;  ◇ - pyrene

Log K'

$10^2$ x (SDS)/mol.dm$^{-3}$

## TABLE 6-1

Retentions and $\alpha$ - selectivities of aromatic hydrocarbons (coding in Appendix 1) at peak maximum concentrations.

CTAB and non-ionic (Tweens) on Silica gel, SDS and T.7 on Alumina packing materials.

| DATUM | CATIONIC SURFACTANT CTAB | NON-IONIC TWEEN | | | ANIONIC | | ODS-HYPERSIL |
|---|---|---|---|---|---|---|---|
| | | 20 | 40 | 60 | SDS | T.7 | |
| $K'_1$ Fluorenone | 2.3 | 5.5 | 7 | 11.44 | 1.18 | 2.83 | 3.7 |
| $K'_2$ Napthalene | 2.7 | 7.16 | 9.3 | 15.45 | 1.90 | 4.5 | 5 |
| $K'_3$ Antracene | 10.7 | 24.3 | 38.8 | 65.71 | 6.9 | 16.00 | 17.3 |
| $K'_4$ Pyrene | 18.5 | 37.66 | 61.5 | 106.28 | 9.33 | 28.25 | 28.3 |
| $\alpha_{1-2} = \dfrac{K'_2}{K'_1}$ | 1.14 | 1.30 | 1.33 | 1.35 | 1.61 | 1.59 | 1.37 |
| $\alpha_{2-3} = \dfrac{K'_3}{K'_2}$ | 4.00 | 3.39 | 4.17 | 4.25 | 3.63 | 3.5 | 3.47 |
| $\alpha_{3-4} = \dfrac{K'_4}{K'_3}$ | 1.73 | 1.55 | 1.58 | 1.6 | 1.35 | 1.76 | 1.63 |

FIGURE   6-2

Variation in retention with Tergitol 7 concentration.
Analytes as in Figure 6-1.
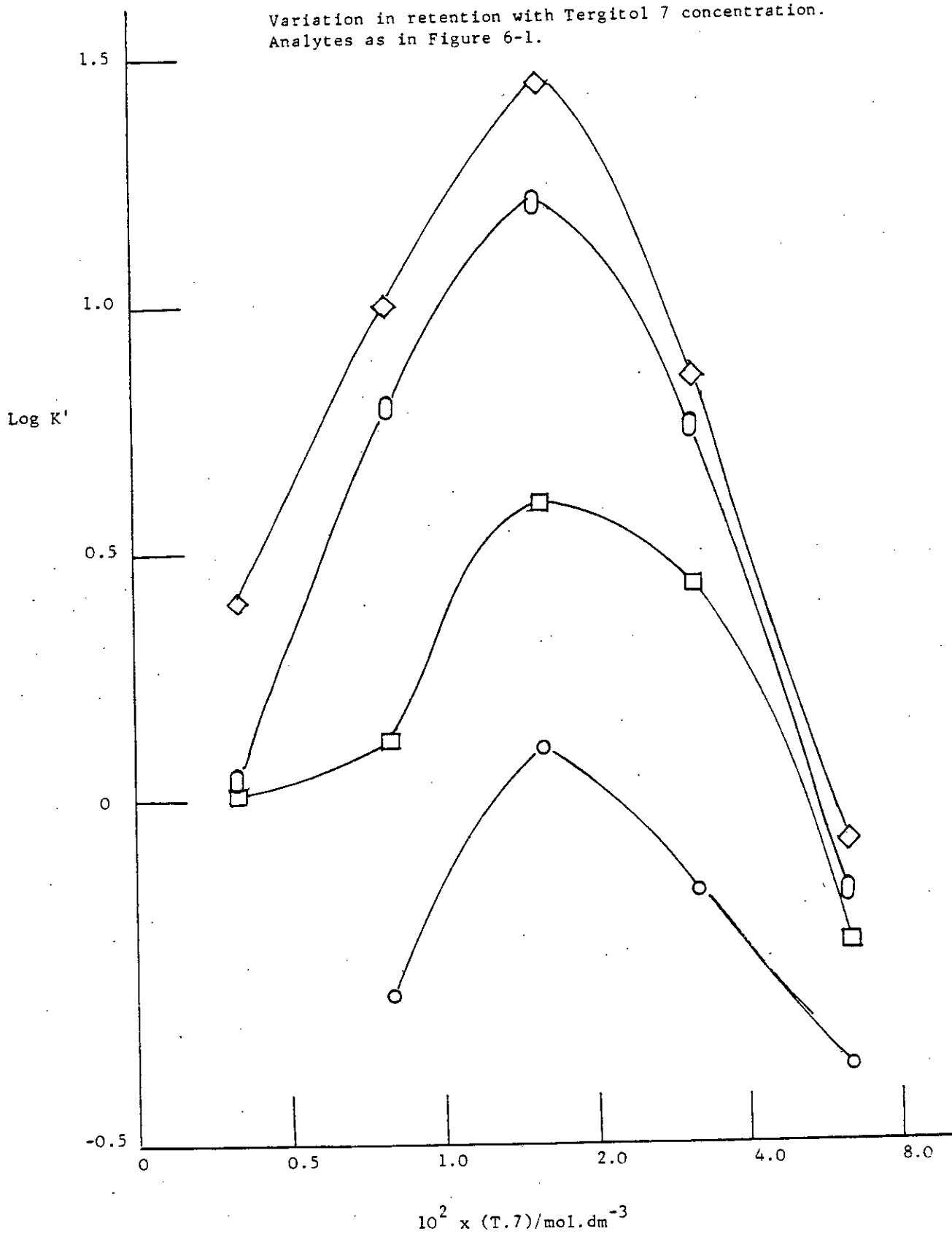
($200-500$ $cm^3$) have to pass through the column before complete

equilibration is achieved at flow rate of $1$ $ml.min^{-1}$. Thus it takes

a long time to equilibrate the column especially with low concentration

of surfactant. Accordingly, if a column is to be prepared for a

practical analytical separation, it is desirable to save working time

by establishment of equilibrium with a concentrated solution before

equilibrating with the final, usually more dilute eluent.

The relationship of capacity factors of some aromatic

hydrocarbons (Appendix 1) to the concentration of SDS in the aqueous

methanolic eluents is shown in Figure 6-1. Although this Figure

indicates that retentions of aromatic hydrocarbons are smaller than

with cationic or non ionic surfactants modified silica gel packings

(101,112) but as Table 6-1 shows, the order of elution, the relative

retention and selectivity of these analytes are similar to those

observed in these earlier studies on columns of alkyl bonded

silica. These observations strongly suggest that there is a common

mechanism of retention, i.e. hydrophobic interaction. Data from

Figure 6-1 shows that as in previous studies, retention of eluites

rises through a maximum (as (SDS) $\simeq$ $3$ x $10^{-2}$ $mol.dm^{-3}$) and then

declines thereafter with increasing solvating power (surfactant

concentration).

6.3.2   Tergitol 7 Effects on Alumina

Figure 6-2 shows the relationship of log K' to concentration

of T.7. Extensive work on the mechanism of IPC by Horvath et al (53)

showed that as the carbon chain length of surfactant increases the
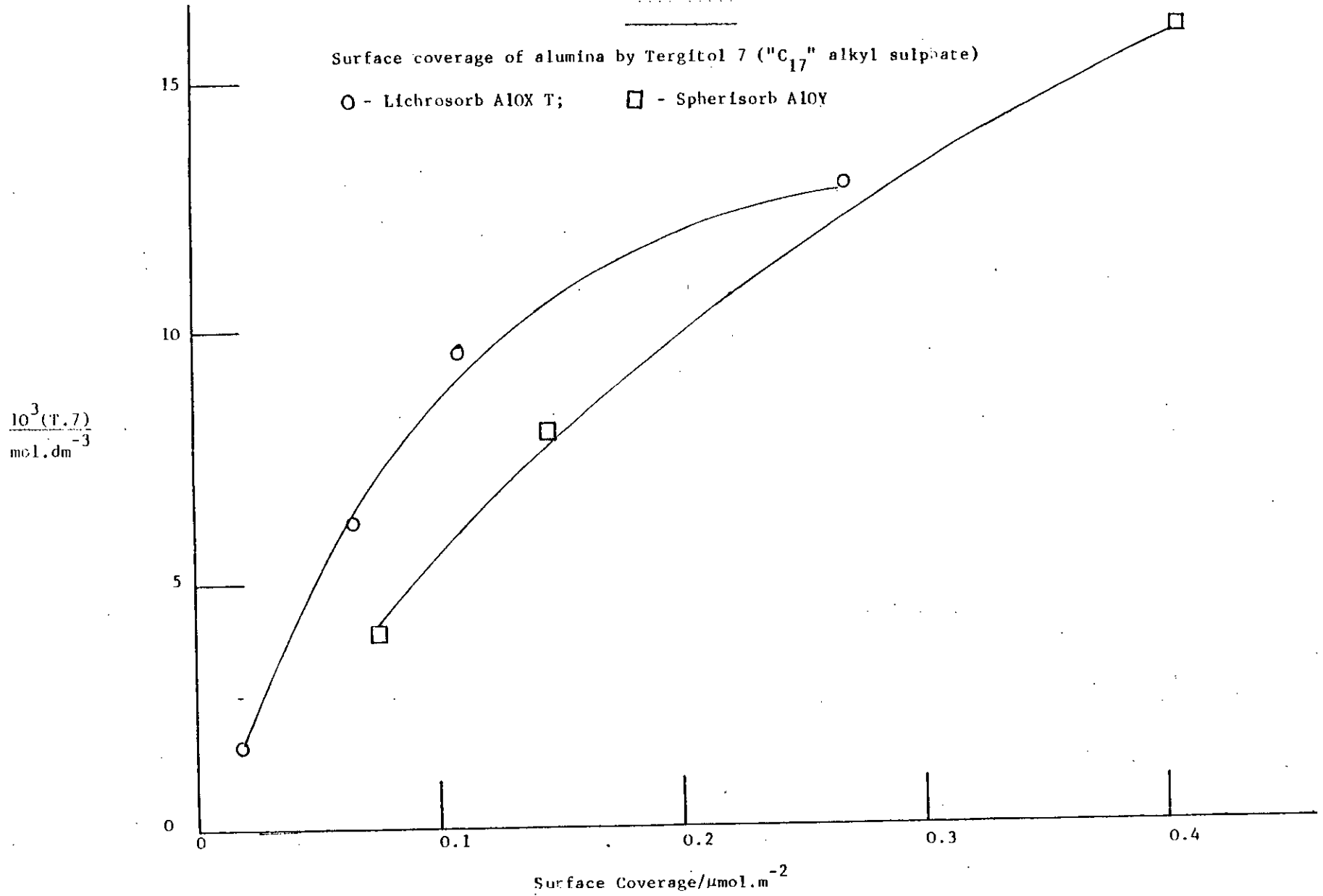
increase in maximum retention^(is) achieved at lower surfactant

concentration. Hemetsberger, et al (98) also explained that

"in reversed phase chromatography using methanol/water as mobile

phase a linear relationship between the capacity ratios and the

amount of (alkyl-bonded) carbon was found in all cases." And

they added that all alkylmethyl phases proved to be superior to

the alkyl phases. Since T.7 is an alkyl sulphate differing only

in chain length from SDS ("$C_{17}$" as opposed to $C_{12}$), an increase

in maximum retention would be expected at lower concentrations

of T.7 than of SDS. This observation can be seen in Figures

6-2 and 6-1, while the peak maximum retention on the SDS systems

was achieved at $3 \times 10^{-2}$ mol.dm$^{-3}$. It is at $1.5 \times 10^{-2}$ mol.dm$^{-3}$

with T.7. In a similar manner Tomlinson et al (86) explained that

when the concentration of surfactant increases capacity ratios

also increase. This effect also can be observed from Figures 6-1

and 6-2.

Selectivity with the T.7 surfactant showed the similarity

between this technique in comparison with ODS-bonded phase and

other earlier techniques (Table 6-1).

## 6.4    Breakthrough Volume Calculations

The amount of surfactant adsorbed on the alumina surface was

calculated both by a modification of the colourimetric breakthrough

technique of Knox and Laird (36) and by a procedure of constant

withdrawal and addition (of aliquots of concentrated surfactant) to

a suspension of alumina in the 1:1 aqueous methanol eluent base.

FIGURE 6-3

Surface coverage of alumina by Tergitol 7 ("$C_{17}$" alkyl sulphate)

O - Lichrosorb Al0X T;        ☐ - Spherisorb Al0Y

It was shown in Chapter 5 that measurement of cationic surfactant concentrations can be achieved with anionic indicators such as sunset yellow (sulphonic acid derivatives). For this study cationic reagent was required: methylene blue was used in these experiments. The relationship of surfactant-dyestuff ion-pair concentration to absorbance ca 655 nm was distinctly non-linear.

Figure 6-3 shows the relationship of surface coverage to T.7 concentration for Lichrosorb Alox T and Spherisorb A10Y. These results agreed with the earlier studies by Rupprecht ( 48) and Scott and Kucera (105) who explained that surface coverage by surfactant is a non-linear function of surfactant concentration in the contacting liquids. The highest degree of sorption measured (at $3.2 \times 10^{-2}$ mol.dm$^{-3}$ (T.7)) showed that there are ca 1.1 $\mu$moles of detergent on each square metre of the alumina surface. The best coverage of silica by covalently bonded mono-layer alkyl chains of equivalent length was shown by Roumelotis and Unger (106) to be roughly three times as dense as that above.

## 6.5    Effect of Salt Concentrations on Capacity Ratios

We have pointed out earlier that the mechanism of retention can be explained as a combination of two distinct physicochemical processes. At low concentrations of surfactant the dominant mechanism is ion-pair formation in the mobile phase, but at high concentrations of surfactant it may be supposed to be an ion exchange process as explained by Wall (102) in relation to double surfactant effects, but in fact the data of Figure 6-4 show some puzzling results.
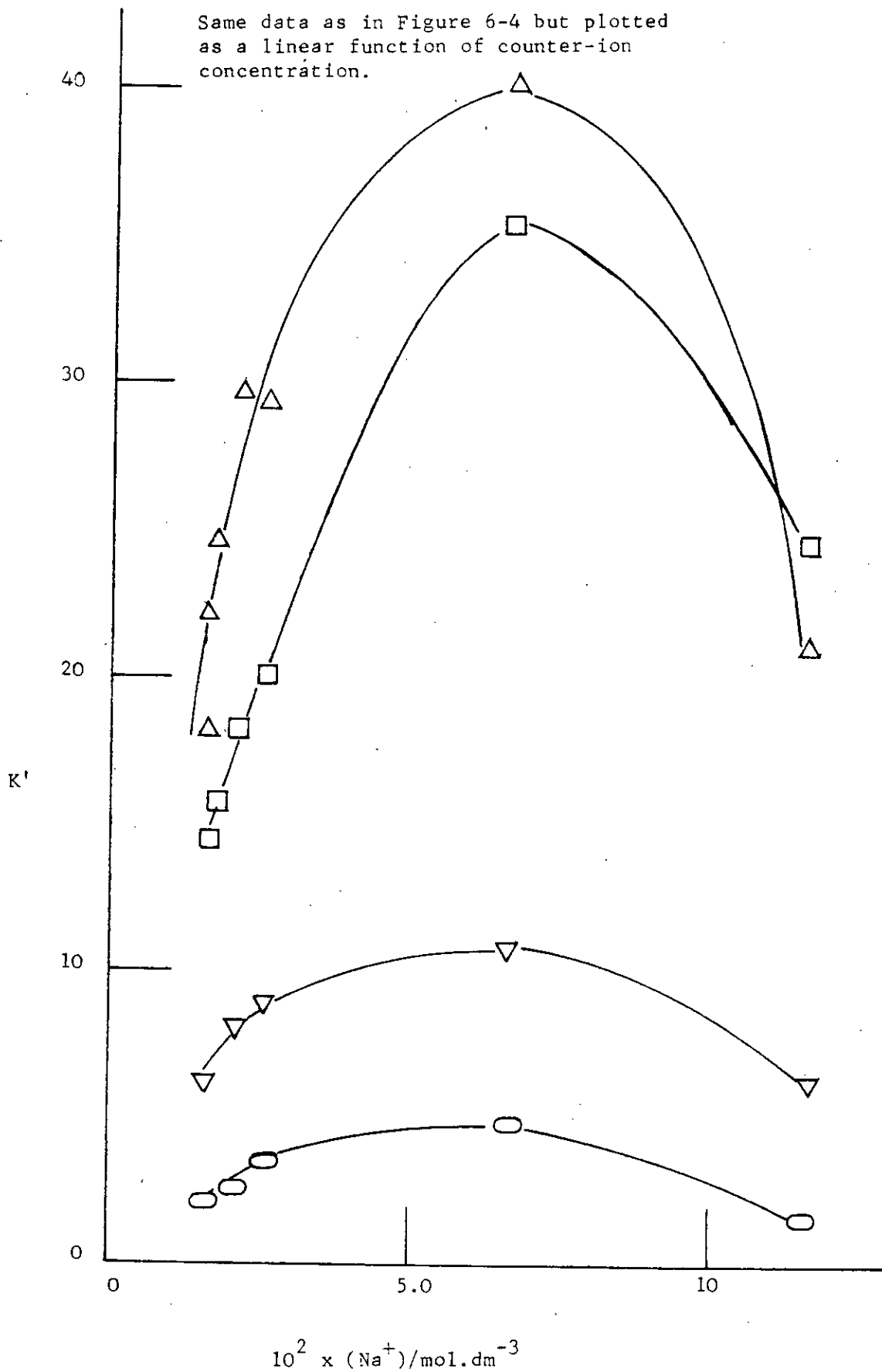
FIGURE 6-4

Effect of change in counter-ion (sodium) concentration on retention at constant ($1.6 \times 10^{-2}$ mol.dm$^{-3}$) concentration of Tergitol 7. ◯ - tyrosine methyl ester; ▽ - 1-amino-1-phenylethane; ◻ - naphthalene △ - 1-amino-1-(1-naphthyl) ethane.

FIGURE 6-5

Same data as in Figure 6-4 but plotted as a linear function of counter-ion concentration.
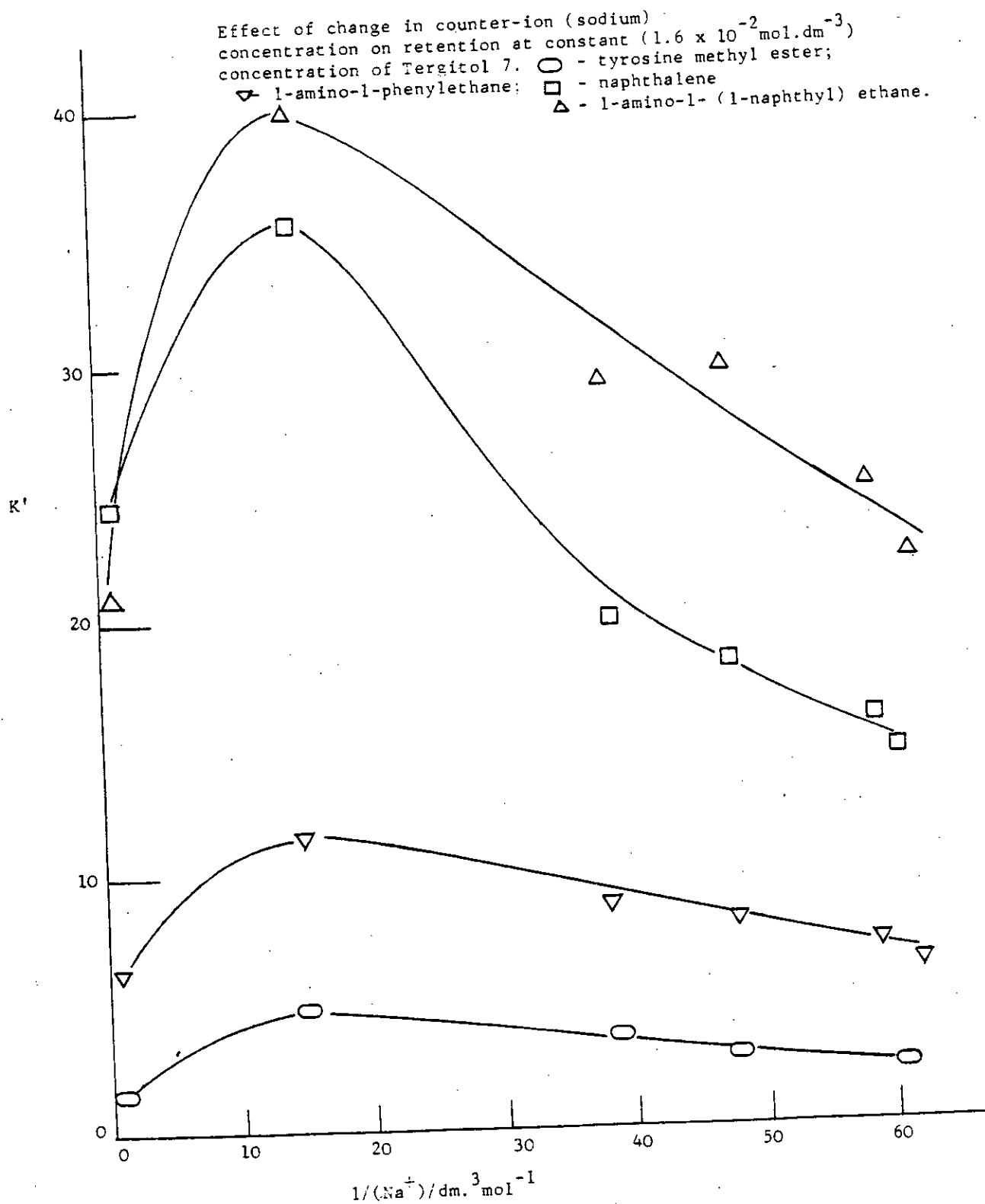
Figure 6-4 indicates the relationship between K' and reciprocal

concentration of counter cation. If the process was only ion

exchange, then a linear relationship would have been observed.

But this curve has a negative slope over most of the concentration

range tested, but reverses to a positive slope at the highest

concentrations. Results obtained with surface active materials

on chemically bonded stationary phase (36) and in earlier work

with non ionic - anionic surfactants on silica (102) clearly

differ from those on the alumina surface. This effect can be

explained as follows: at high concentration of surfactant these

relatively hydrophobic ammonium ions (or their ion pairs with alkyl

sulphate anions) are retained primarily by hydrophobic interaction

with the hydrocarbon side chains of surfactant molecules sorbed on

the alumina surface. This conclusion is borne out by comparison

of the data given in Figure 6-5 with the similar plot given as

Figure 4 of the earlier study (102). Clearly the changes in

retention in this present study with increasing salt/are similar
concentration

for both non polar and ammonium ion analytes up to ca $5 \times 10^{-2}$

mol.dm$^{-3}$ (Na$^+$), but the large decrease in retention power at even

higher salt concentration was not observed in the earlier studies

on the silica - non ionic - anionic surfactant system. However,

these two sets of data are not strictly comparable since the oxide-

surfactant systems and contacting liquids are not the same.

6.6     Effect of pH in Solute Retention

Failure to explain the mechanism of retention as nearly pure

FIGURE 6-6

Effect of change of pH on retention at constant counter
ion and Tergitol 7 concentrations.  Analytes as in
Figure 6-4 except ◯ - tryptophan methyl ester.
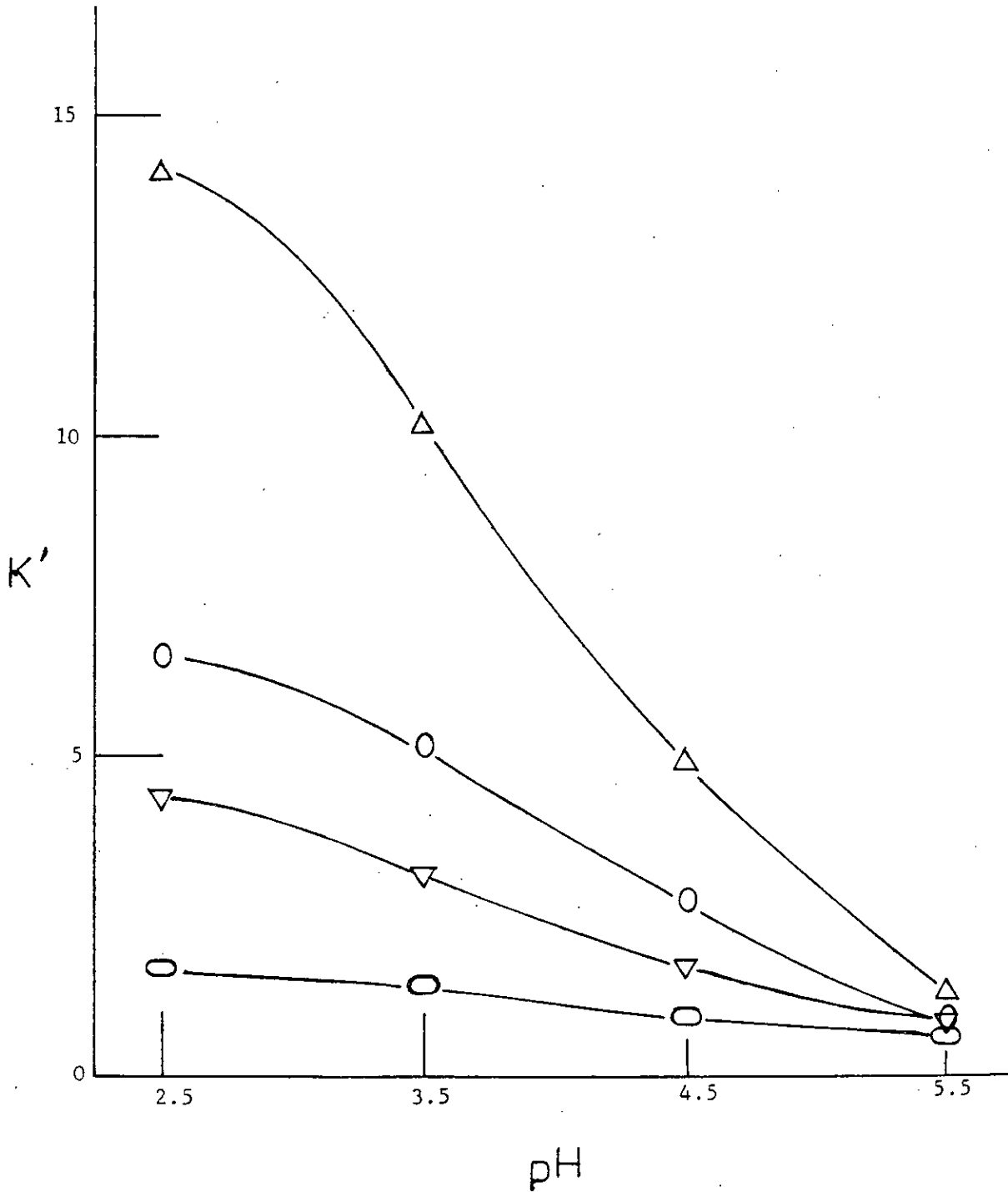
ion exchange from the above discussion, led us to investigate the effect of pH on solute retention. The variation of pH was achieved by addition of mineral acid, $H_3PO_4$, to a solvent system in which methanol, surfactant, and sodium ion concentrations were held constant, so that all variables except $(H^+)$ and ionic strength were controlled. Hamilton, et al (107, 14) in a series of similar experiments showed that the capacity ratios of cationic analytes (amino acids) on polystyrene sulphonic acid exchangers/steadily decreased as the pH of the eluent increases from 2-6. Figure 6-6 shows exactly similar results obtained in our experiments.

Results from Figure 6-6 show that the effects of pH like the effects of salt concentration have no simple explanation, while the ammonium ion analytes of this present study are at least 99% in the ionised form over the whole of the observed pH range. However, this behaviour is typical of the pattern observed with classical ion exchangers, and retention in the present system is accordingly best described as a balance of ion exchange and hydrophobic bonding. If the density of alkyl chains on the alumina surface was proportional to $(H^+)$, that could account for the results described by Figure 6-6. No data on the pH sensitivity of anionic surfactant binding to alumina are presently available so more investigation should be done to justify this assertion.

## 6.7  Application of Anionic Detergents on Dynamically Coated Stationary Phase

Application of this novel technique of dynamically coated stationary phase using anionic surfactants on basic aluminium 111

FIGURE 6-7

Specimen separation of a mixture of neutral, unionised analytes. Column packed with Spherisorb AlOY, 125 x 4.6mm; flow rate $1cm^3$/min of 1:1 methanol/water containing $3.2 \times 10^{-2}$ mol.$dm^3$ SDS (pH adjusted to 3.0 by addition of phosphoric acid). Analytes in order of elution are acetone, 2,3-xylenol, 9-fluorenone, and naphthalene.
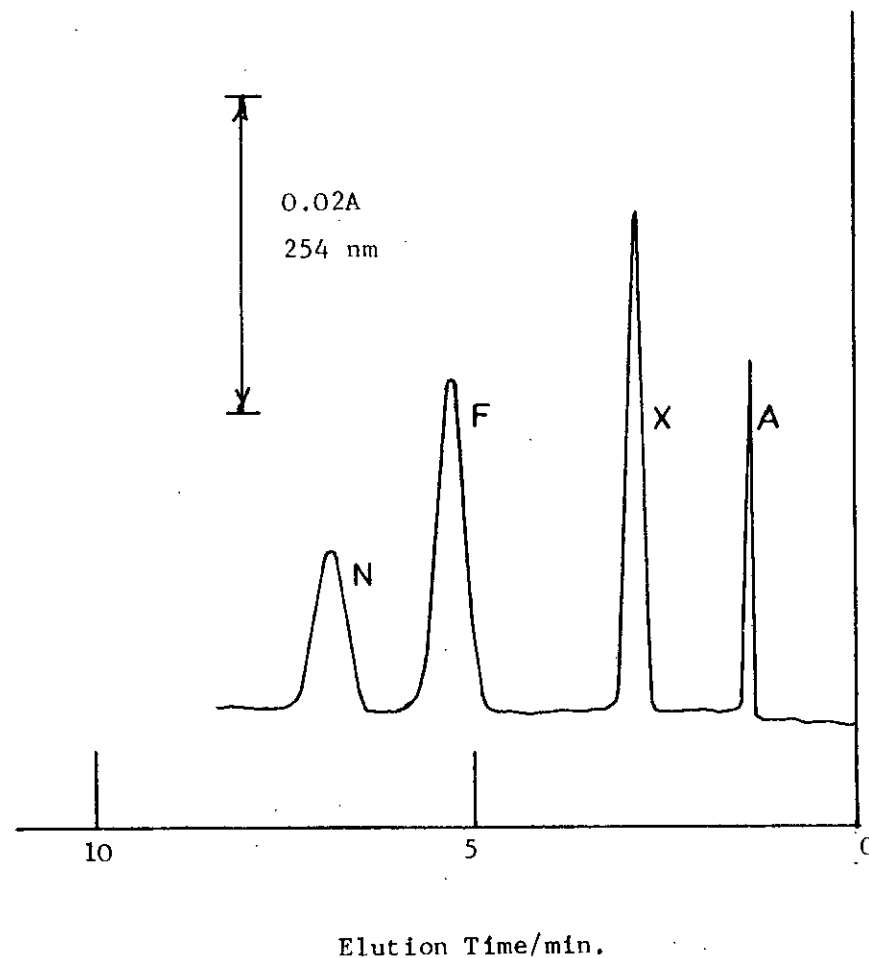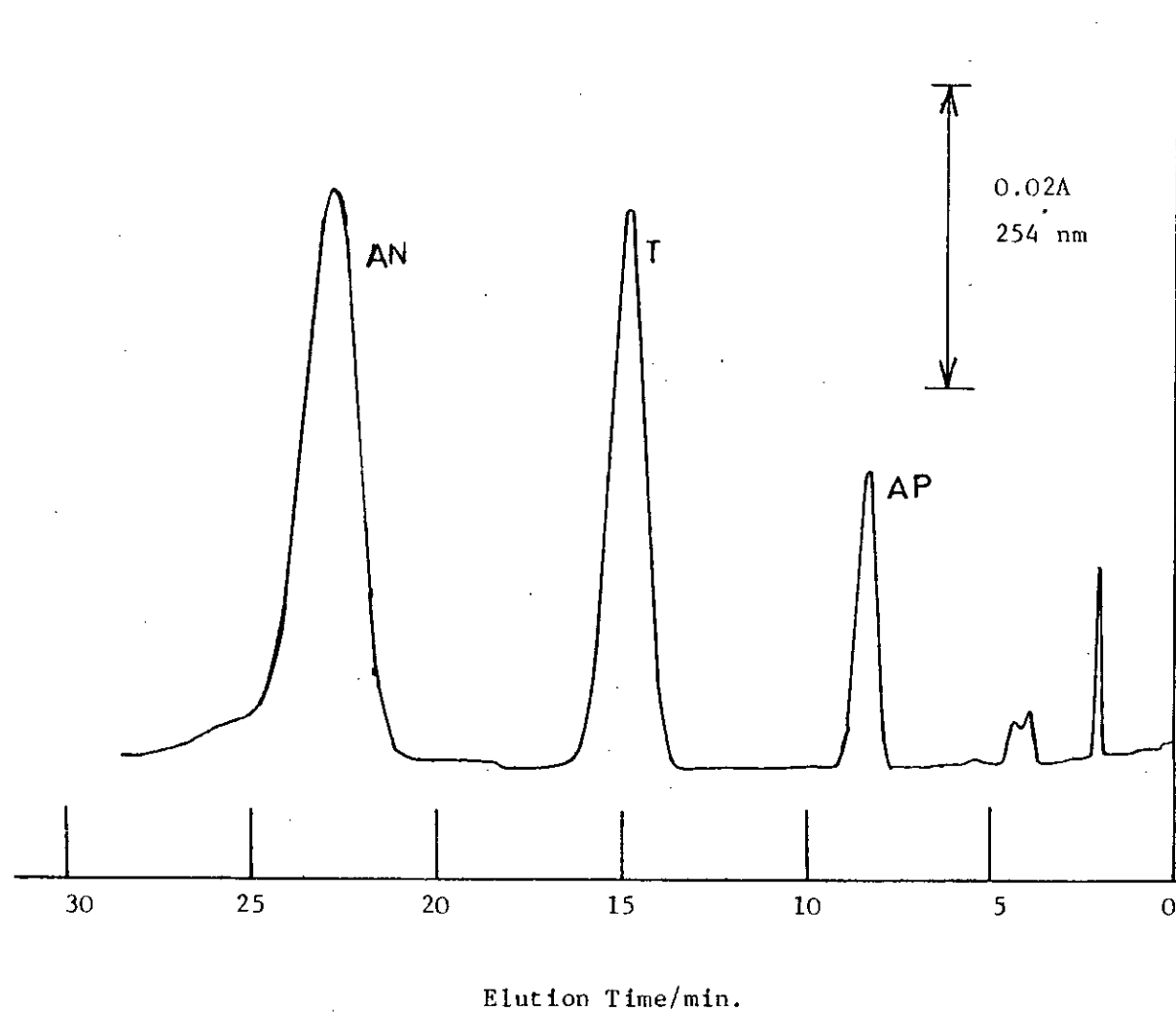
0.02A
254 nm

Elution Time/min.

FIGURE 6-8

Specimen separation of cationic analytes on same column as in Figure 6-7. Eluent as in Figure 6-7, but analytes in order of elution are; three impurities, 1-amino-1-phenylethane, tryptophan methyl ester, and 1-amino-1- (1-naphthyl) ethane.

oxide gel is shown in Figures 6-7 and 6-8. Figure 6-7 is a record

of a specimen separation of four neutral analytes on an alumina-SDS

column. This figure indicates that cationic analytes may be well

retained (under control by variation of pH, surfactant concentration,

counter-cation concentration, and organic modifier concentration of

the eluent system) in operating conditions such that acidic,

zwitterionic, and neutral analytes will also be retained (according

to their hydrophobic binding capacities). Separation of tyrosinyl

peptides in the earlier study by Wall (102) on dynamic ion exchange

suggested that similar separations can be obtained with the same

analytical capabilities, but as was indicated earlier in this Chapter,

the retention mechanisms are different in these two procedures, hence

their selectivities for a given analysis will also differ.

To obtain the optimum efficiency of separation for desired

solutes of Figure 6-7, a series of operations was carried out at

different eluent flow rates, i.e. 1.0, 0.8, 0.6, 0.4 and 0.2 $cm^3.min^{-1}$.

These experiments showed that as the speed of elution decreases from

1 to 0.2 $cm^3.min^{-1}$ the column efficiency increases steadily (the number

of theoretical plates increased from 3000 to 4000 over this eluent

flow range for the unretained acetone peaks and from 750 to 3000 for

the fluorenone peak). If the approximation and calculation of reduced

parameters as outlined by Knox (43) and estimation of diffusion

constants by the Wilke-Chang equation (108) are taken into account, then optimal

column efficiency for fluorenone would be suggested to be at an eluent

flow rate of 0.1 $cm^3.min^{-1}$. However, the above data shows that this

newest mode of oxide gel-surfactant LC is capable of giving

separation efficiencies comparable to those obtained with more

conventional techniques.

Figure 6-8 shows the separation of three fully protonated

amines with the same column/eluent system used to produce Figure 6-7.

From the discussion in the previous Chapter, it is clear that

there should be no retention of ionisable eluites in the absence of

surfactant in the aqueous methanol eluent.

Experimental observation showed that there is no interaction between

alumina oxide gel and protonated cationic eluites, and indeed the

three cationic eluites would be little retained on columns of

alkyl-bonded silica from this mobile phase (methanol/water) at pH3.

The observation of pronounced retention of quaternary ammonium

analytes as well as of aralkyl ammonium ions suggests that although

a purely ion exchange mechanism cannot be supported (cf. Figure 6-4)

for these ionic eluites, there must be a substantial element of ion

exchange or ion pair partition in the retentive processes.

6.8    Conclusions

The present investigation confirms the potential and

versatility of a new approach to hydrophobic ion exchange

chromatography based on dynamic generation of a retaining surface

on porous oxide gel column packing materials.  Moreover, it has shown

that the surface layer(S) generated by direct (electrostatic)

interaction of an anionic surfactant with a basic aluminium 111

oxide differs significantly from the product of an acidic silicon 1V

oxide and mixed non ionic-anionic surfactants.

Chromatographic systems based on those described above are readily set up, and are clearly versatile, since analyte retention is controlled (primarily) by eluent composition variables, i.e. pH, ionic strength, organic modifier type and concentration, and surfactant type and concentration. Since the mechanism of retention of cationic analytes appears to have both hydrophobic and ion exchange components, variation of operating temperature should alter selectivity of separation as well. Further study is required before a greater understanding of the detailed mechanism(s) of analyte retention in this new "dynamic" mode of liquid chromatography can be attained, but its value as an additional tool in the analytical workshop is apparent.

CHAPTER   7

Hydrophobic Chromatography with Dynamically Coated

Stationary Phase: Non Ionic Surfactant Effects

7.1     Introduction

It was shown in previous Chapters that retention of charged

analytes on columns packed with hydrophilic oxide gel particles can

be observed when small amounts of charged surfactants are dissolved

in the eluting solvent. These adducts to the mobile phase in this

technique are often charged surfactants such as alkyl sulphonates and

sulphates or alkylammonium salts. There is little information available

on the use of non polar surfactants in chromatography on packed columns

of alkyl bonded silica or silica gel.

The previous study showed that only cationic surfactants reacted

with acidic gel surfaces (e.g. $SiO_2$ or $ZrO_2$) so that hydrophobic inter-

:actions with anionic and neutral aromatic compounds could be observed

(101).

In this Chapter the effects of addition of some non ionic

surfactants to aqueous methanolic eluents of silica columns are

described. This class of surface active compound is also shown to be

deposited onto the silica gel surface. Furthermore, data presented

here shows that as the hydrophobicity of the surfactant increases (i.e.

as the hydrophobic carbon chain length increases), retention of aromatic

hydrocarbons and ketones also increases.

Another interesting aspect of the use of non ionic surfactants

is the separation of amines, amino acids and short peptides on columns

packed with silica by the secondary addition of <u>anionic</u> surfactants

to aqueous methanolic solvent systems. Wall (102) showed that the

mechanism of interaction in this mode of chromatography is probably

ion exchange between cationic analytes and the sulphonic acid end of

the anionic surfactant which is hydrophobically attached to the non

ionic surfactant bound to the gel surface. Excellent separation

efficiency was reported for this mode of chromatography. The use of

double surfactants is discussed in Chapter 8.

Appendix 3 indicates the approximate formula and molecular

weight of the Tween materials used in the following experiments.

7.2        Measurement of Adsorbed Surfactant

Colorimetric estimation of non ionic surfactants did not appear

to be a practical means of measurement for surface reaction estimations,

therefore, measurement of adsorbed surfactant was done by a gravimetric

method. In this procedure samples (ca 1 gr.) of silica gel (GA 43,

$dp = 9 \ \mu m$, $S_{BET} \simeq 170 \ m^2 gr^{-1}$) were weighed into tared, stoppered

flasks and dried in vacuo for 30 minutes at $120^{o}C$. Flask and contents

were cooled in a desiccator and reweighed to determine the dry weight

of silica. Aqueous methanol (1:1, V:V, 200 $cm^3$) containing a known

concentration of surfactant was mixed with the dried silica for 5 minutes

in an ultrasonic cleaning bath to ensure complete dispersion of the

particles. The mixture was filtered by suction through a sintered glass

funnel and the excess solvent carefully sucked from the retained silica.

The filter-dry silica was weighed and then dried in vacuo at $120^{o}C$,

cooled in a desiccator and weighed again to obtain both the dry weight

and the "wet" weight.  In no case was the amount of detergent adhering
to "filter-dry" silica as excess solution greater than a few percent
of the amount bound via surface interaction.

The above procedure was repeated for each determination with a
fresh 200 cm$^3$ sample of surfactant solution to ensure complete
equilibration.

Table 7-1 gives the results of these experiments and shows that
the surfactant adsorption on the silica surface decreased with increasing
chain carbon number.

<u>TABLE   7-1</u>

<u>Sorption of Tween on GA43 Silica Gel</u>

| Surfactant | (Tween)/ g.dm$^{-3}$ | Tween Ads/ $\dfrac{mg}{gr.SiO_2}$ | Hydrophobic Alkyl Chain |
|---|---|---|---|
| Tween 20 | 2.0 | 123 | $- (CH_2)_{11} CH_3$ |
|  | 3.0 | 197 |  |
| Tween 40 | 2.1 | 65 | $- (CH_2)_{15} CH_3$ |
|  | 4.3 | 150 |  |
| Tween 60 | 2.2 | 52 | $- (CH_2)_{17} CH_3$ |
| Tween 80 | 5.0 | 85 | $- (CH_2)_8 CH = CH(CH_2)_7 CH_3$ |

Furthermore, Table 7-1 also shows that as the concentration of
surfactant rises in the contacting solution surface adsorption also
increases.  This effect was described by Rupprecht ( 48).  The maximum

FIGURE 7-1

Relationship of log K' to concentration of Tween 20
in 1:1 methanol:water eluent. Column packed with
Hypersil (dp $\simeq$ 5 μm). Flow rate 1 $cm^3.min^{-1}$. at
ambient temperature. Eluites: as coding in Appendix 1.
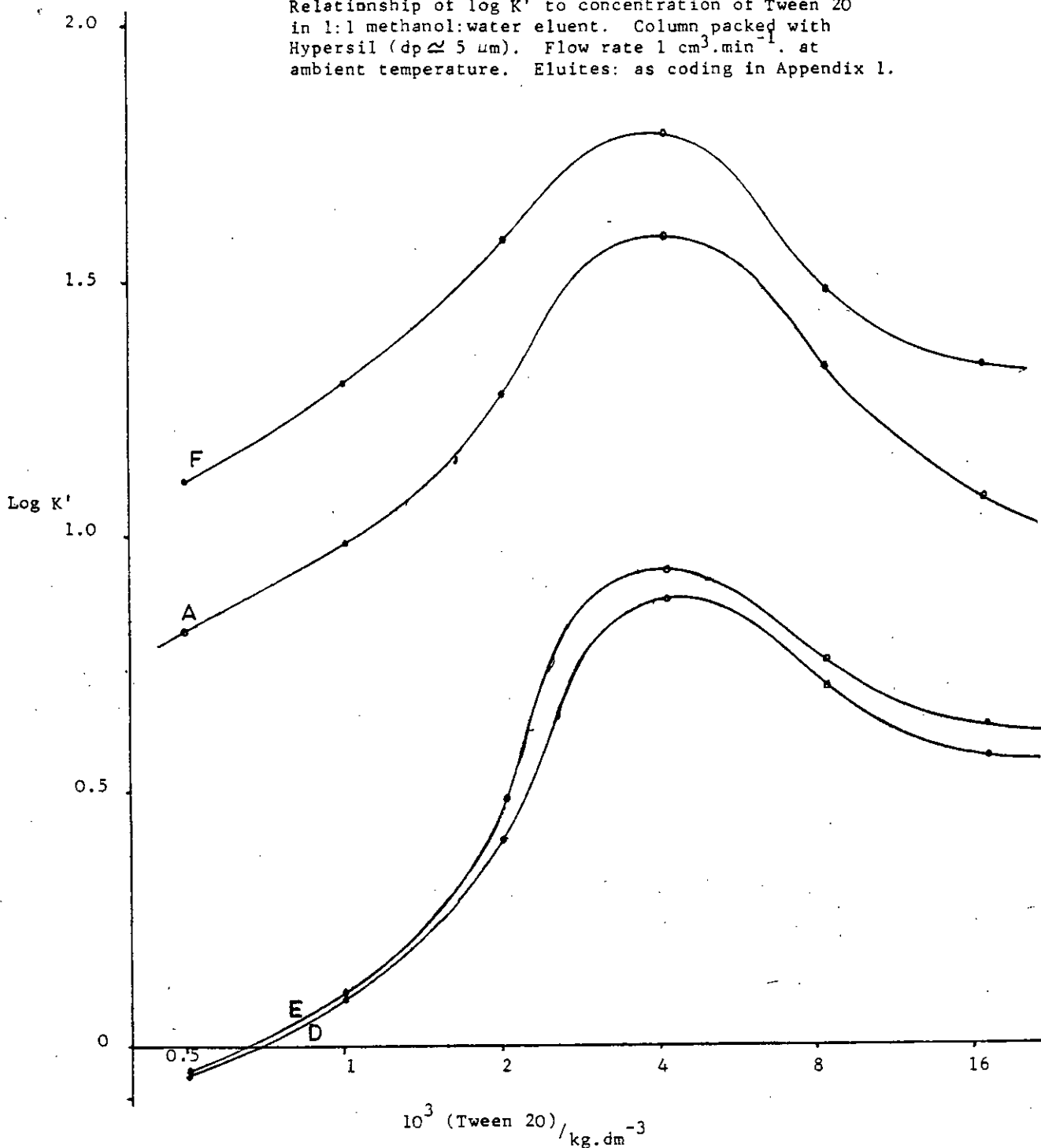
FIGURE  7-2

Demonstration of minimal retention of sample mixture
of Figure 7-1 on Hypersil from 1:1 methanol:water
solvent system in absence of surfactant.



Elution Time/min.
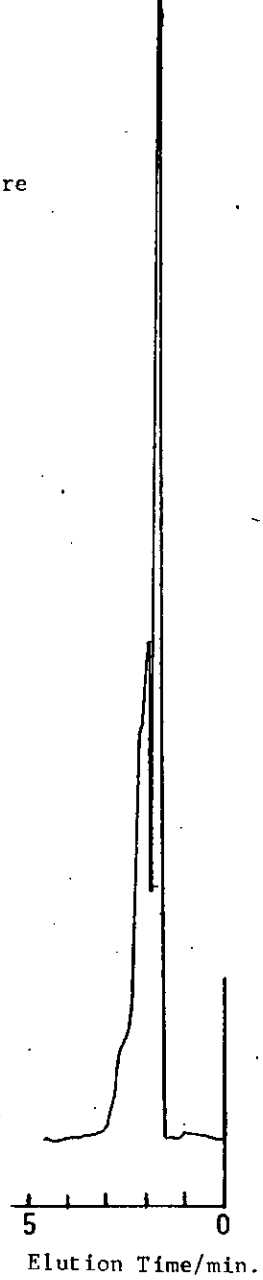
adsorption was achieved by Tween 20, that is at $3 \times 10^{-3}$ Kg .dm$^{-3}$ in which 197 mg of surfactant was adsorbed on 1 gr. of silica. This corresponds to surface coverage of about 1.5 $\mu$ mol.m$^{-2}$ and is about half the value reported by Roumelotis and Unger (106) for "state of the art" $C_{18}$ and $C_8$ alkylbonded silicas.

## 7.3    Separation of Aromatic Hydrocarbons on Silica/Tween 20

A silica column was equilibrated with a mobile phase consisting of water and methanol in the ratio of 1 to 1 with addition of the appropriate amount of Tween 20 to give concentrations ranging from (0.5 to 16) x 10$^{-3}$Kg.dm$^{-3}$. All separations were carried out at ambient temperature (15-23$^{\circ}$C) with an eluent flow rate of 1 cm$^3$.min$^{-1}$. The aromatic hydrocarbons and Ketone used in this Chapter were indicated in Appendix 1.

Experimental data were measured with the column of Hypersil (125 mm x 4.6 ID.) after passage of between 100 and 150 cm$^3$ of solvent containing Tween 20. Figure 7-1 shows the dependence of capacity factor, K', of analytes on the Tween 20 modified silica gel column as a function of detergent concentration. There is no retention of aromatic hydrocarbons in the absence of Tween 20 as is shown in Figure 7-2. Capacity ratio initially increases as the concentration of surfactant increases, and then declines with further increase of (Tween 20) above the $3 \times 10^{-3}$ Kg.dm$^{-3}$level. The order of elution of aromatic hydrocarbons and Ketones also depends upon their hydrophobic nature. The more lipophilic the analyte, the greater is its retention.

Comparison of the retentions of aromatic hydrocarbons in the

FIGURE   7-3

Chromatogram of aromatic hydrocarbons (as coding in Appendix 1 ;
K: Acetophenone) on column of Figure 7-2 in equilibrium with a
solution of Tween 20 (0.81 x $10^{-2}$ kg.$dm^{-3}$) in 1:1 methanol:water.
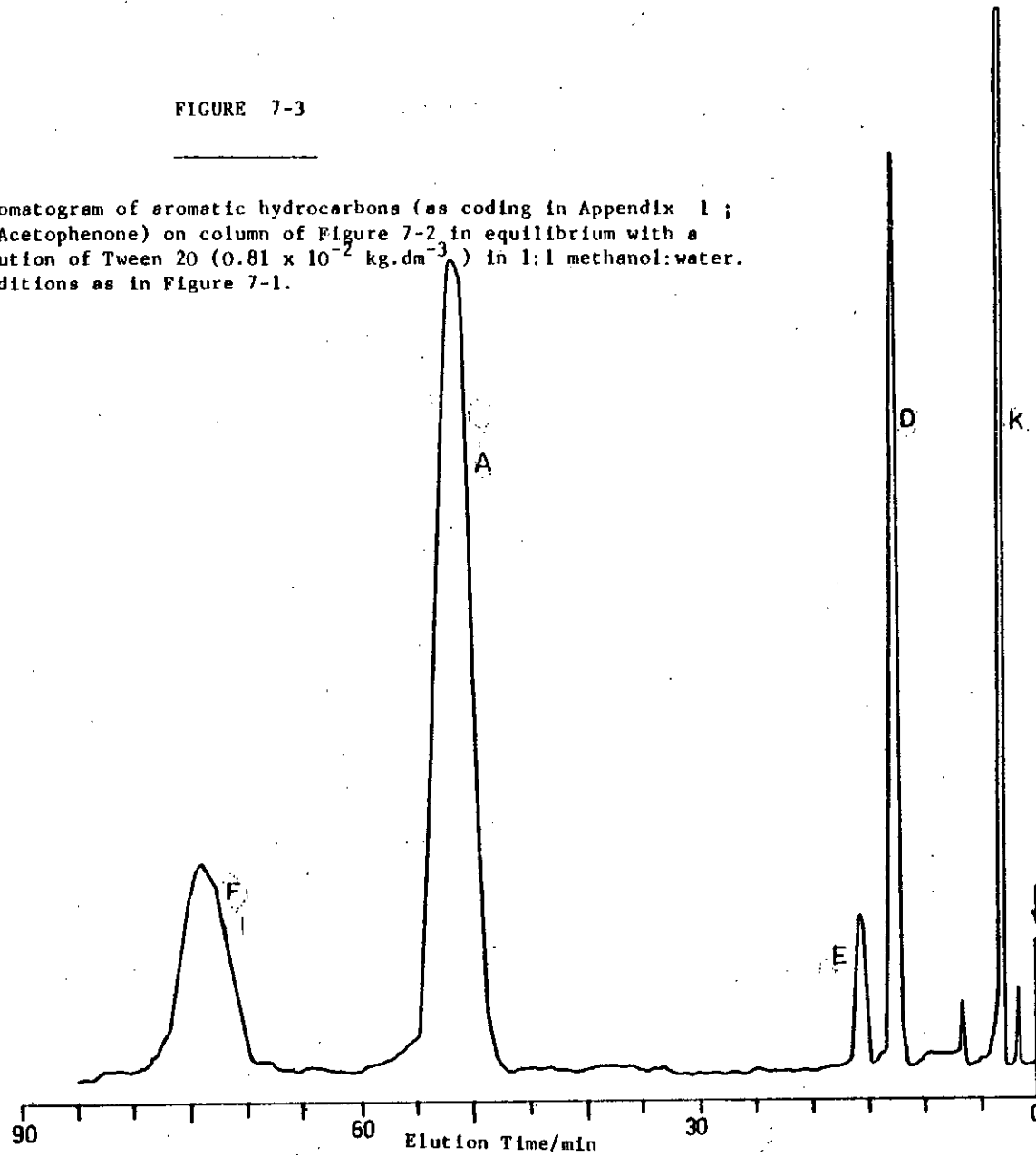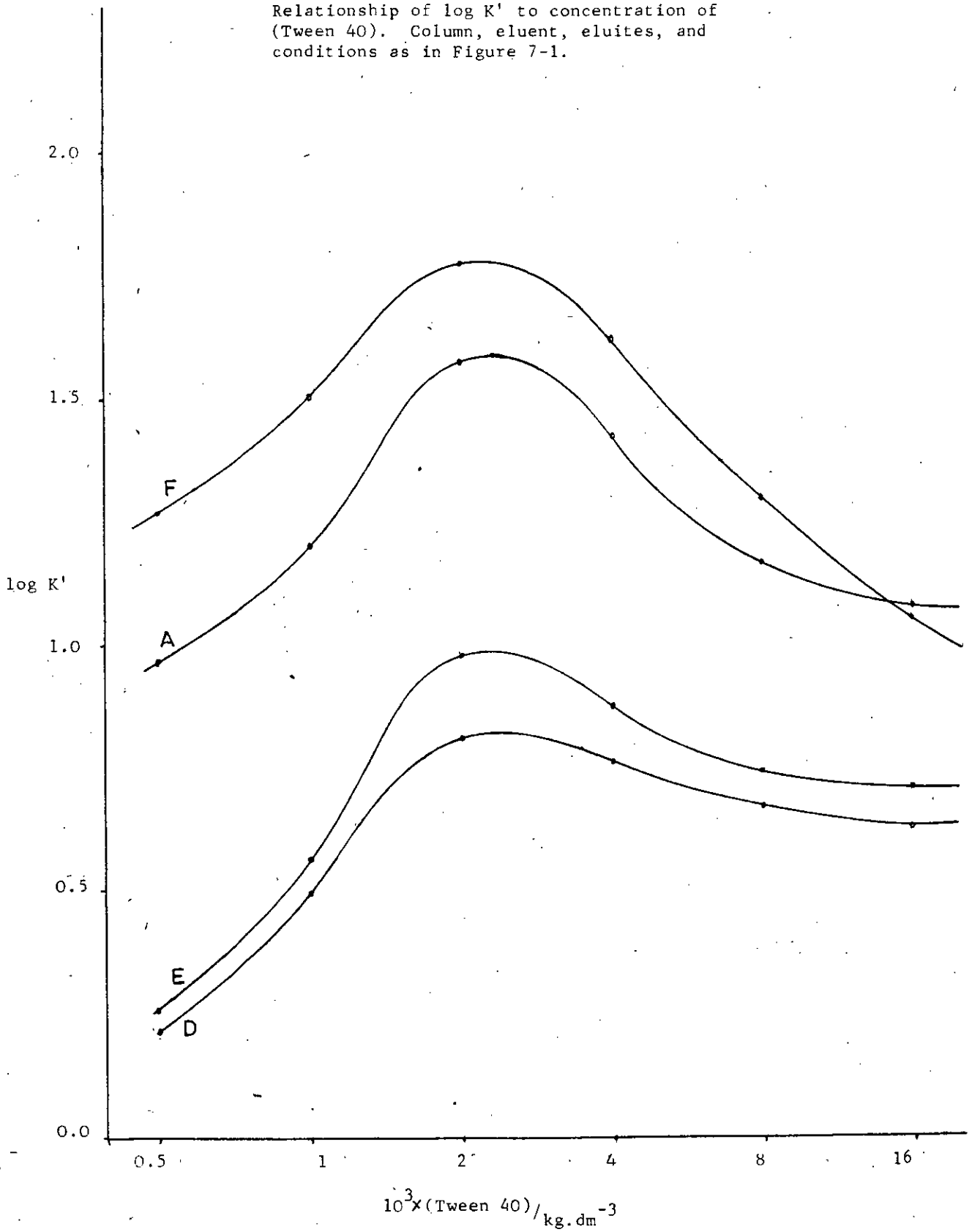Conditions as in Figure 7-1.

FIGURE 7-4

Relationship of log K' to concentration of
(Tween 40). Column, eluent, eluites, and
conditions as in Figure 7-1.

present work and those from previous investigations on silica packing

modified by cationic surfactant (101) and on the alumina modified by

anionic surfactant (110) show large absolute increases in the action of

non-ionic surfactant. For example, the maximum capacity ratio for pyrene

in the present work is about 59, while maximum retention from Figure 5-6

for pyrene is about 15 and on alumina was 25 (when Tergitol 7 was used).

This observation suggests that surface coverage is more complete with

non-ionic surfactant. A representative chromatogram of aromatic

hydrocarbons and aromatic Ketone is presented in Figure 7-3.

7.4     Separation of Aromatic Hydrocarbons and Ketone on Silica
        Using Tween 40

A 1:1 mixture of methanol/water containing 0.5 $\rightleftharpoons$ 16 x 10$^{-3}$

Kg.dm$^{-3}$ of Tween 40 was employed in these experiments. Tween 40 has

a 16 carbon hydrophobic alkyl chain (4 carbons longer than Tween 20),

and gave greater retention of hydrocarbons than Tween 20.

Figure 7-4 shows the relationship between K' and (Tween 40).

Maximum retention of hydrocarbons was observed at 2 x 10$^{-3}$ Kg.dm$^{-3}$

(Tween 40). The order of elution was the same as with Tween 20/silica

columns.

Passage of 75-100 cm$^3$ of solvent containing Tween 40, was

sufficient for column equilibrium.

It was pointed out above that the mechanism of solute retention

in this mode of chromatography is a consequence of the dynamic interaction

between surface and surfactant. This interaction for Tween materials

should consist, at least partially, of hydrogen bonding between surfactant

and
/surface. If this is true then it should be equally applicable to basic

FIGURE 7-5

Relationship of log K' to concentration of (Tween 40) in (1:1 v/$_v$) methanol:water eluent. Column packed with Alumina (dp $\cong$ 10$\mu$m). Conditions as in Figure 7-1.

Eluites: O - 9-fluorenone;  □ - naphthalene;
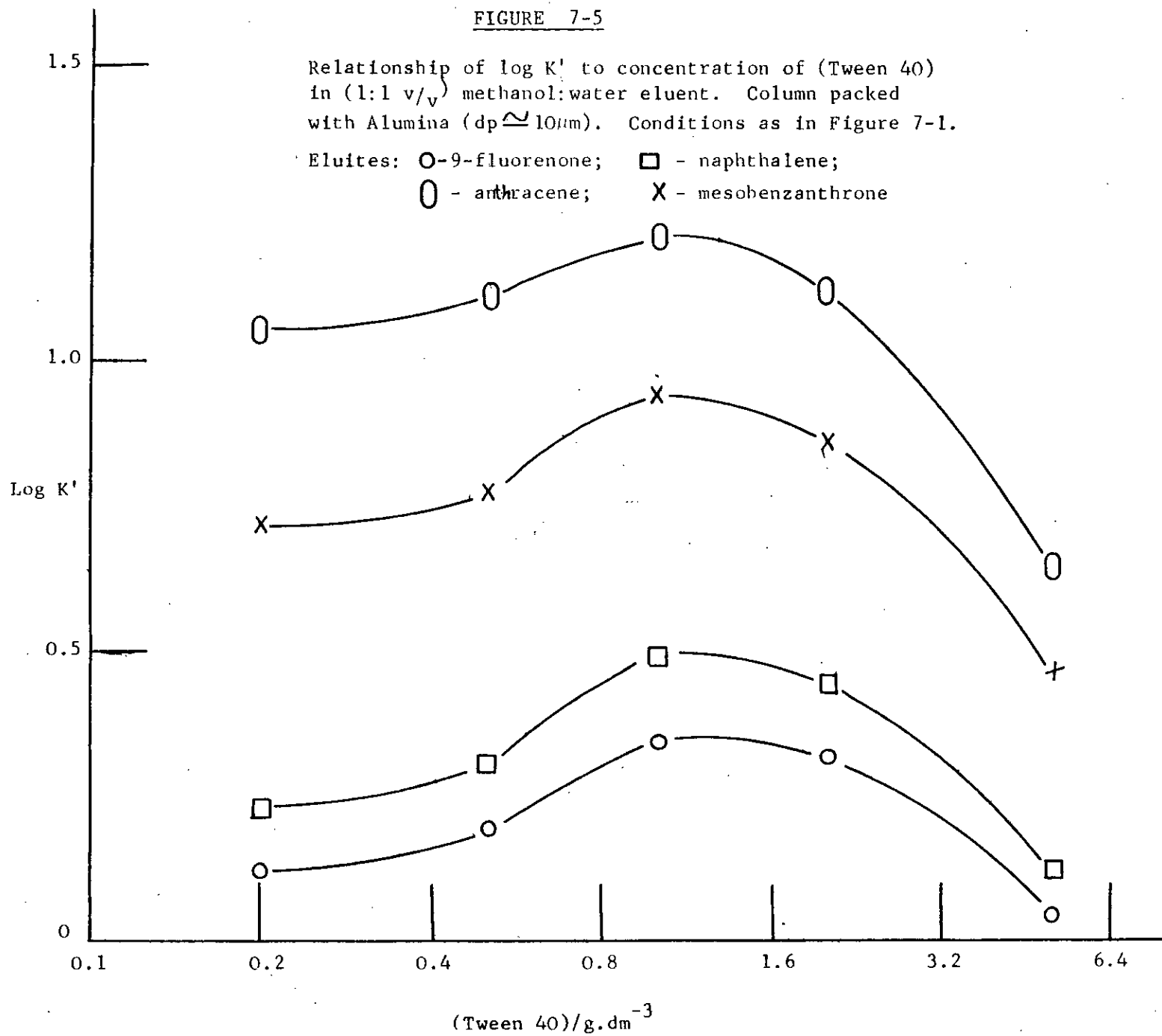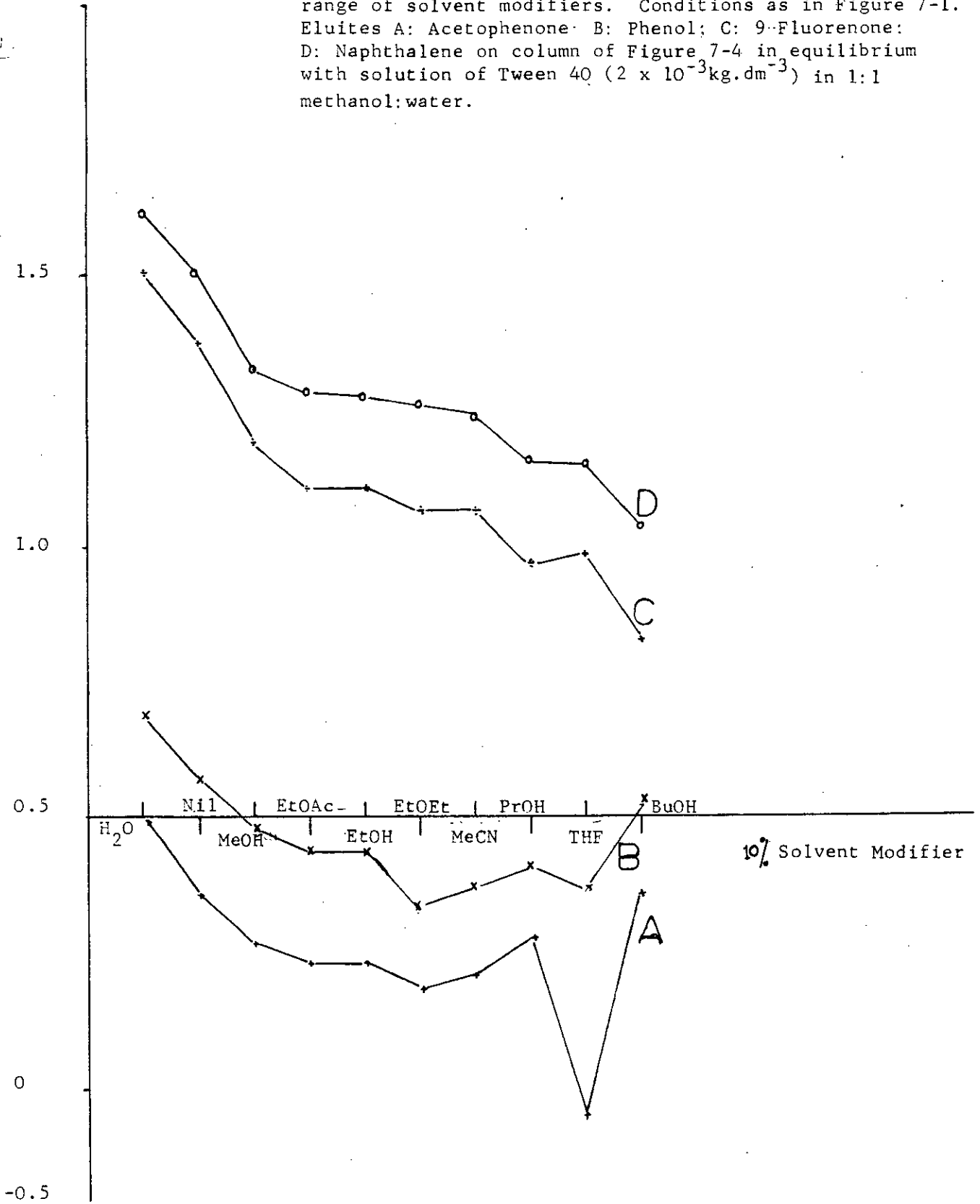         O - anthracene;    X - mesobenzanthrone

(Tween 40)/g.dm$^{-3}$

FIGURE 7-6

Relationship of log K' to 10% (V:V) addition of a range of solvent modifiers. Conditions as in Figure 7-1. Eluites A: Acetophenone B: Phenol; C: 9 Fluorenone: D: Naphthalene on column of Figure 7-4 in equilibrium with solution of Tween 40 ($2 \times 10^{-3}$kg.dm$^{-3}$) in 1:1 methanol:water.

| | | | |
|---|---|---|---|
| NIL | = No addition | MeCN | = acetonitrile |
| MeOH | = methanol | PrOH | = propan-1-ol |
| EtOAc | = ethyl acetate | THF | = tetrahydrofuran |
| EtOH | = ethanol | BuOH | = butan-1-ol |
| EtOEt | = diethyl ether | | |

FIGURE   7-7

Separation of fluorenone (C) and naphthalene (D) on column of Figure 7-2.
Eluent composition as in Figure 7-6.  Less retained eluites are acetophenone (A)
and phenol (B), respectively.



Elution Time/min.
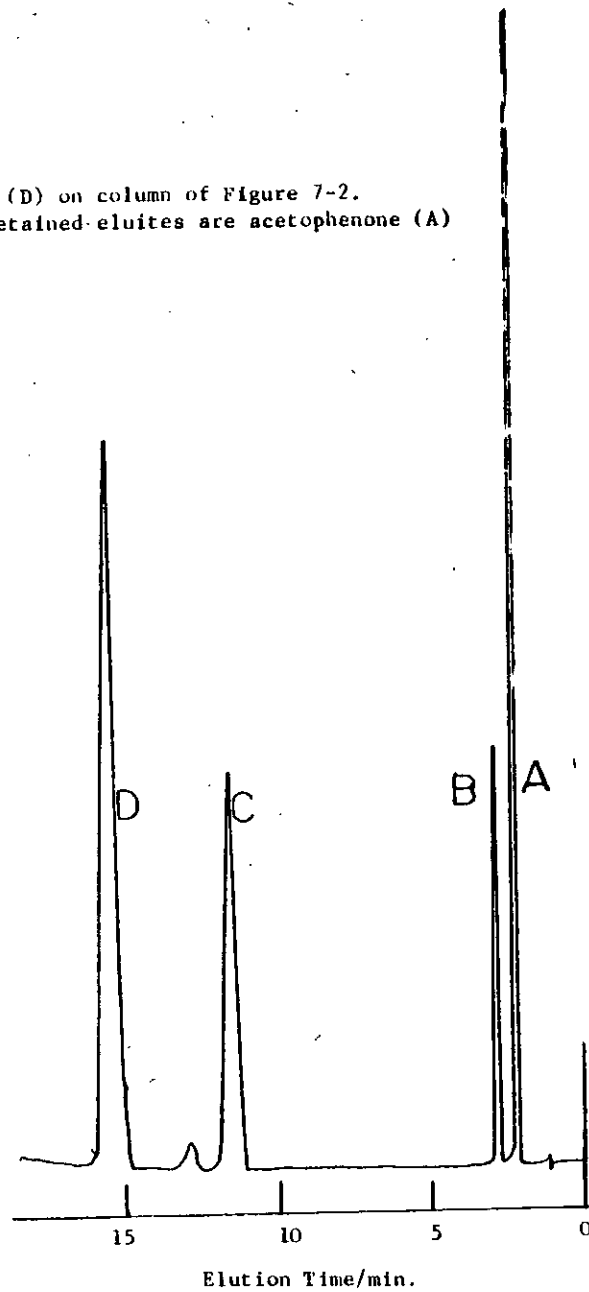
packing materials such as alumina. Therefore a 10μm alumina (Al0Y

alumina listed in Table 5-1) was packed and equilibrated with eluent

containing Tween 40. Figure 7-5 represents the relationship between

K' and (Tween 40) on the alumina packings. Alumina adsorbents are

usually considered to have a more reactive, polar surface than those

based on silica gel and perhaps residual surface polarity reduces

the tendency of hydrophobic analytes to be retained at the Tween 40/

Alumina interface. Certainly this range of eluites (and several others

not shown) yields K' values about half of those measured on silica/

Tween 40 column systems. However, the surfactant concentration at

which maximal retention is observed is essentially the same as that

found in the silica column study.

Control of both absolute and relative retention of several

different eluites is usually maintained by choice of organic modifier

and its concentration in "reversed phase" chromatography. Karger et al

(111) have shown that selectivity in this liquid chromatographic mode

is critically dependent on the nature and proportion of organic modifier

in the eluent system. Figure 7-6 shows the effect of 10% additives on

solvent composition (45:45 methanol/water) on capacity ratio. As the

organic characteristics of solvent additives increase, the capacity

ratios decrease. This is analogous to observations on bonded phases but

the effects of solvent changes are by no means equal on all four solutes.

Clearly, control of organic modifier nature and proportion allows

considerable control of selectivity in any separation attempted in this

new HPLC mode.

Excellent separation of non-polar aromatic hydrocarbons can be

seen in Figure 7-7. Figure 7-2 shows that there is no retention of these

FIGURE 7-8

Separation of acetophenone (A), benzene (B), toluene (T) and nitrobenzene (N).
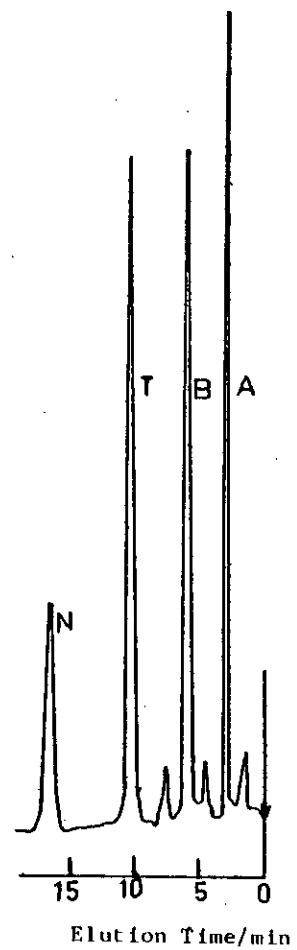Column, eluent, and condition as Figure 7-7.



Elution Time/min

FIGURE 7-9

_____

Separation of phenol (P), para-cresol (C), 3, 5 xylenol (X),
and phenetole (pH) on a column (125 mm x 4.6 mm. ID) of
ODS-Hypersil (dp≈ 5 μm). 6:4 (V:V) methanol:water was passed
through the column at ambient temperature at 1 $cm^3.min^{-1}$.



Elution Time/min
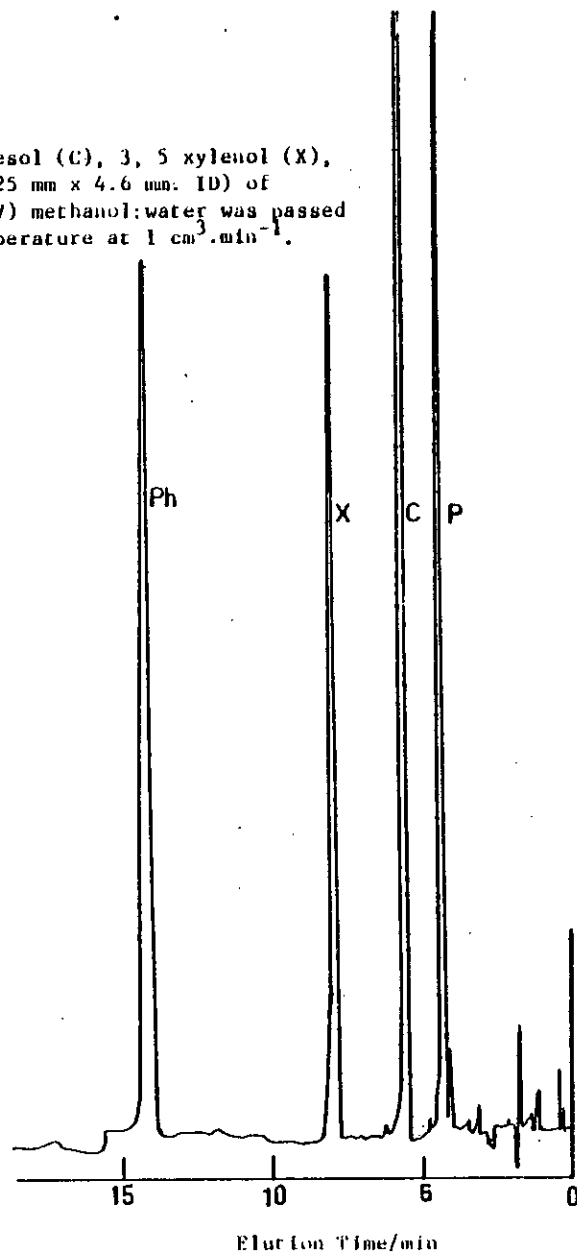
## FIGURE  7-10

Separation of sample mixture as
shown in Figure 7-9 with the solvent
system as in Figure 7-7.
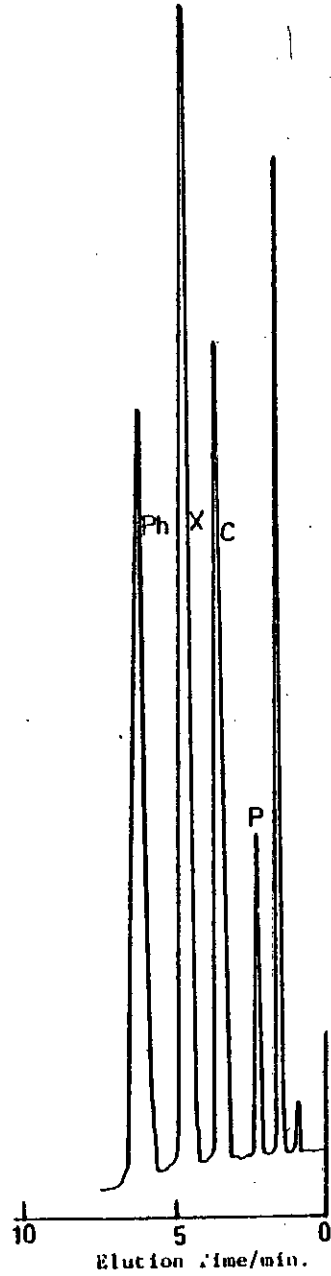
FIGURE   7-11

Relationship of log K' to concentration of Tween 60.
Column, eluent, eluites and conditions as in Figure 7-1.
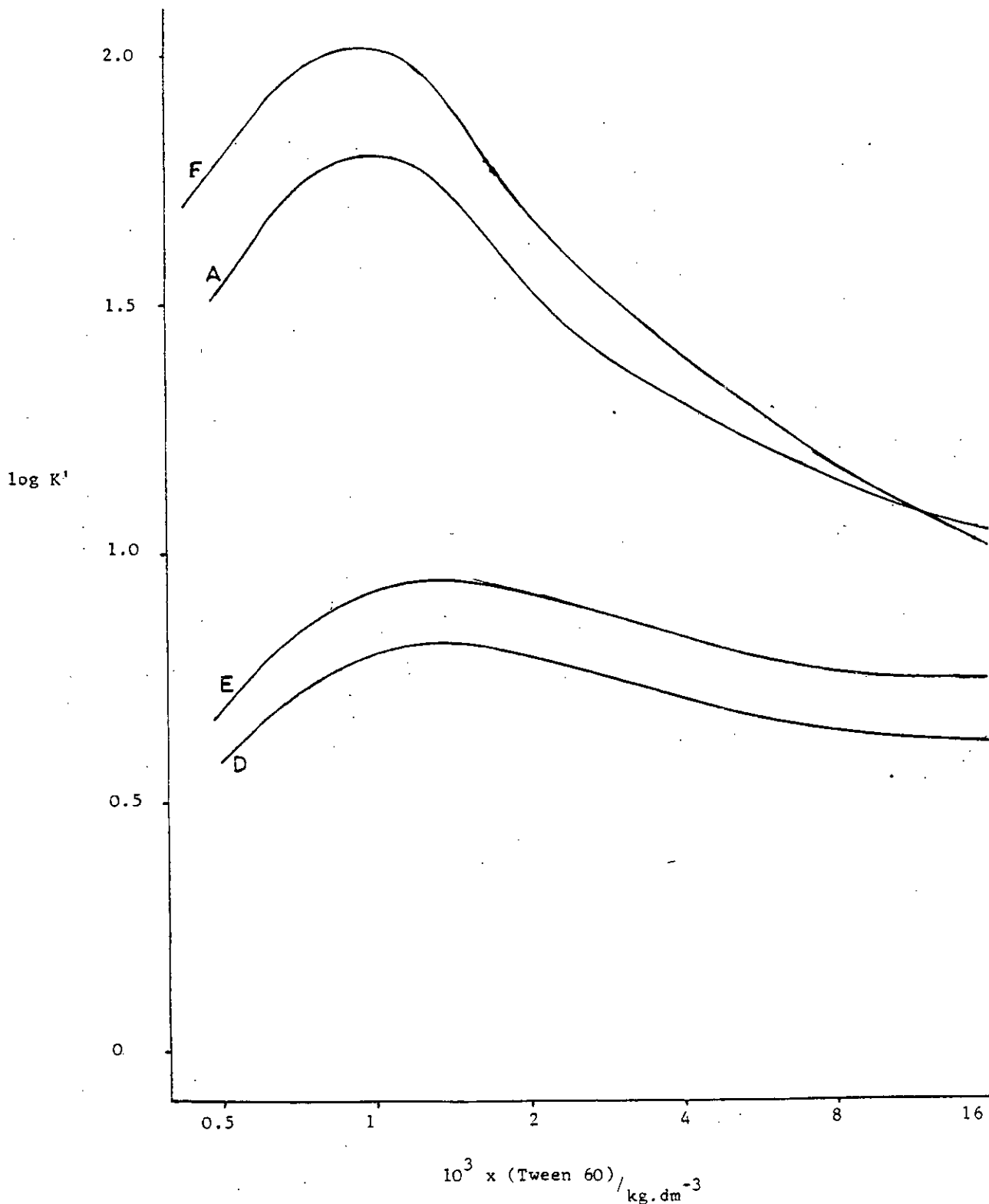
FIGURE  7-12

Separation of acetophenone (A), fluorénone (F), naphthalene (N), and anthracene (An) on column of Figure 7-2 in equilibrium with a solution of Tween 60 ($1.1 \times 10^{-3}$ kg.dm$^{-3}$) in 1:1 methanol:water.
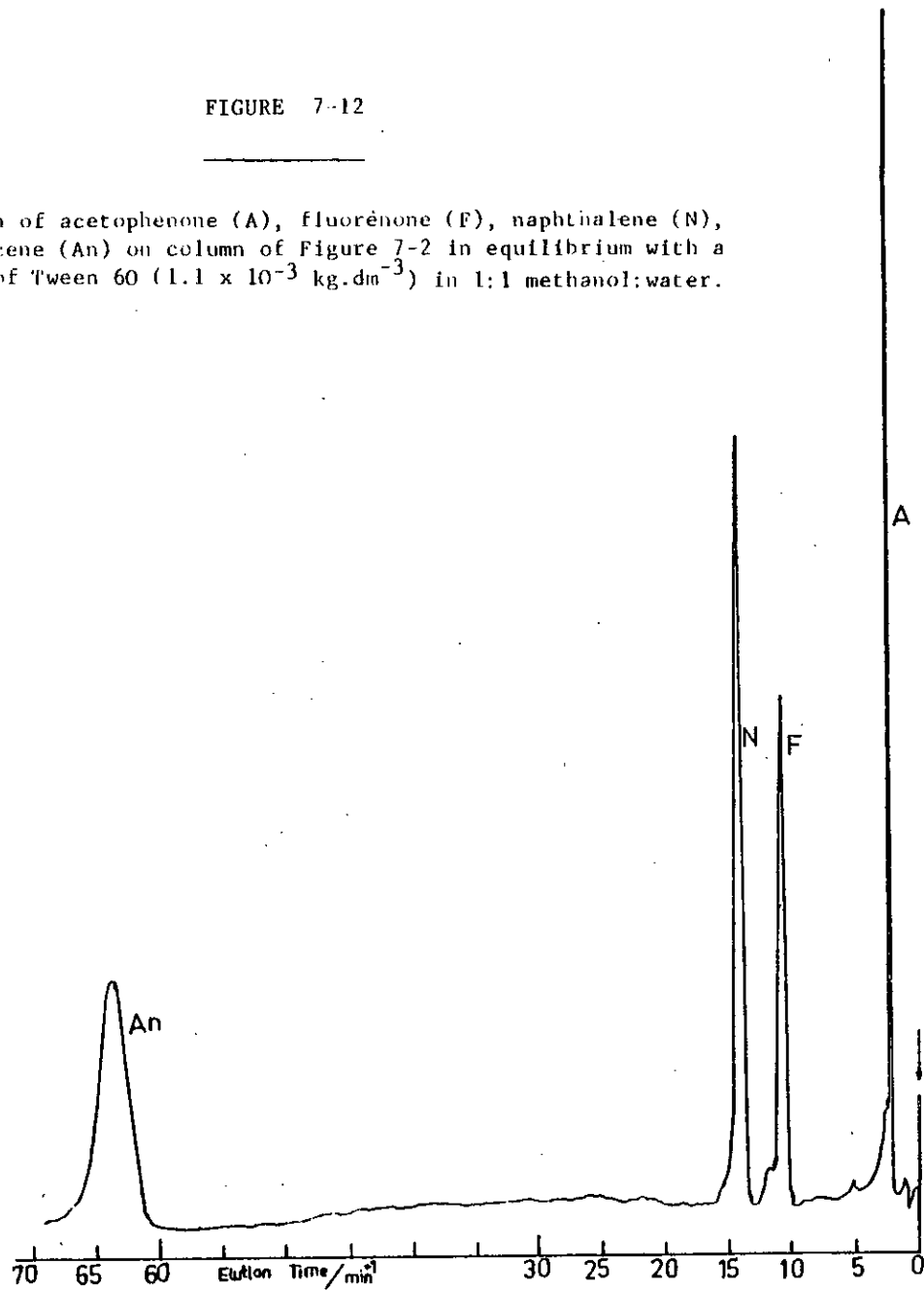
analytes on silica in the absence of Tween. Addition of small amounts of surfactants (i.e. $2 \times 10^{-3}$ Kg.dm$^{-3}$ ) of Tween 40 to the aqueous methanol eluent allows even very similarly retained analytes such as fluorenone and naphthalene and also phenol and acetophenone to be separated with sufficient resolution.

Figure 7-8 shows a comparable separation of another test mixture (i.e. acetophenone, nitrobenzene, benzene and toluene) at $2 \times 10^{-3}$ Kg.dm$^{-3}$ of Tween 40.

Separation of one of the standard test mixtures suggested by Knox (37) for determination of the efficiency of columns packed with alkyl bonded silica is shown in Figure 7-9 and the separation of the same mixture on a dynamically coated stationary phase is shown in Figure 7-10. The order of elution and relative retention of analytes on the ODS-hypersil column (Figure 7-9) is very similar to that observed in aqueous methanolic Tween 40 elution from the silica column (Figure 7-10)

## 7.5    Separation of Hydrocarbons with Tween 60 as Eluent Additive

Tween 60 has an 18 carbon chain and is the most hydrophobic surfactant in the series of (saturated alkyl) Tweens used in these experiments. Table 7-1 shows that surface modification can be achieved at lower concentrations of Tween 60 than of Tween 20 and Tween 40.

Figure 7-11 shows that maximum hydrocarbon retention is at ca $1 \times 10^{-3}$ Kg.dm$^{-3}$ of Tween 60. Capacity ratios of analytes also increase slightly when Tween 60 is employed in place of Tweens 20 and 40. A typical chromatogram of non-polar aromatic hydrocarbons is shown in Figure 7-12 as an example of the use of Tween 60.

FIGURE 7-13

Relationship of log K' to concentration of Tween 80
in eluent. Eluites:as in Figure 7-3.

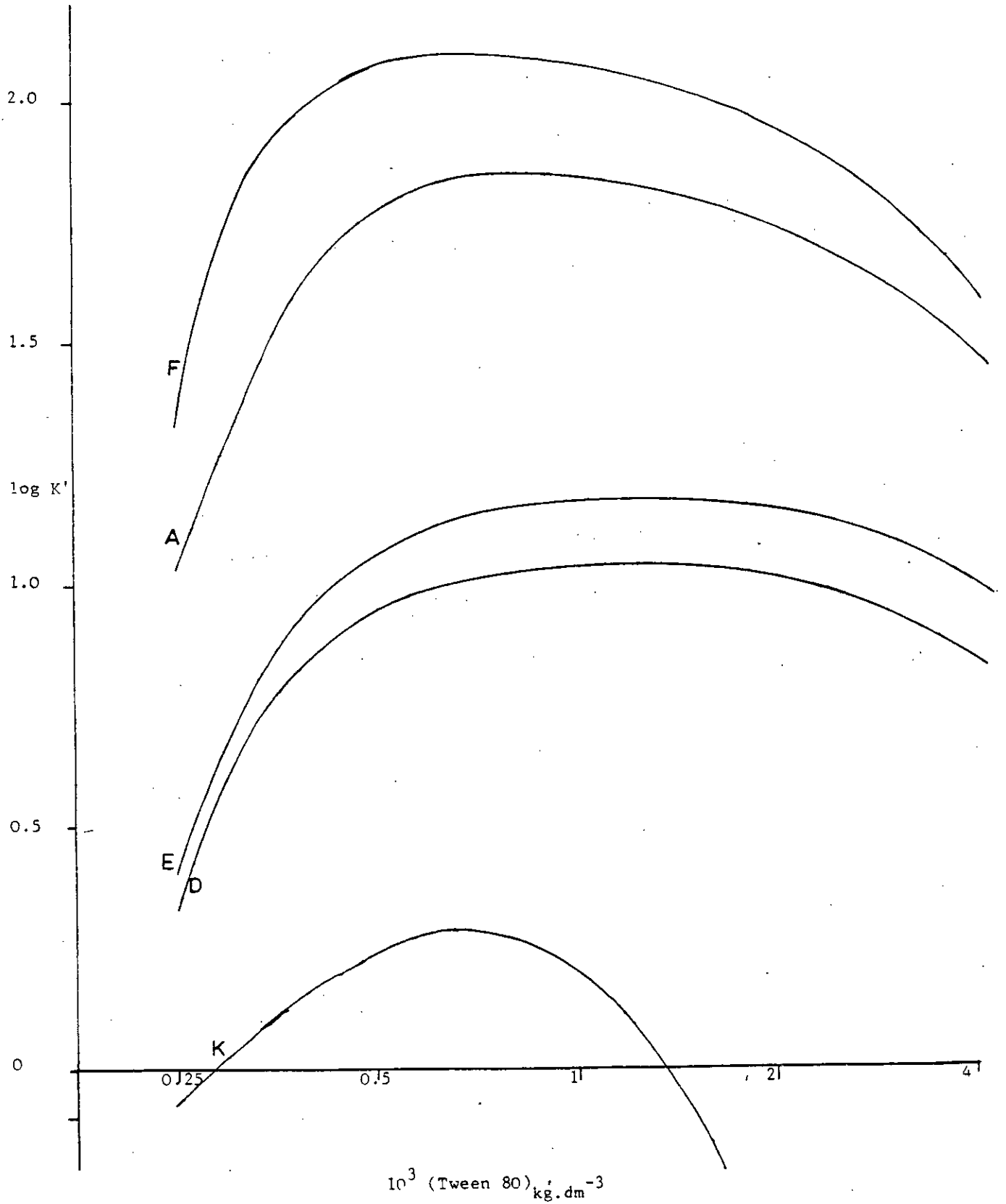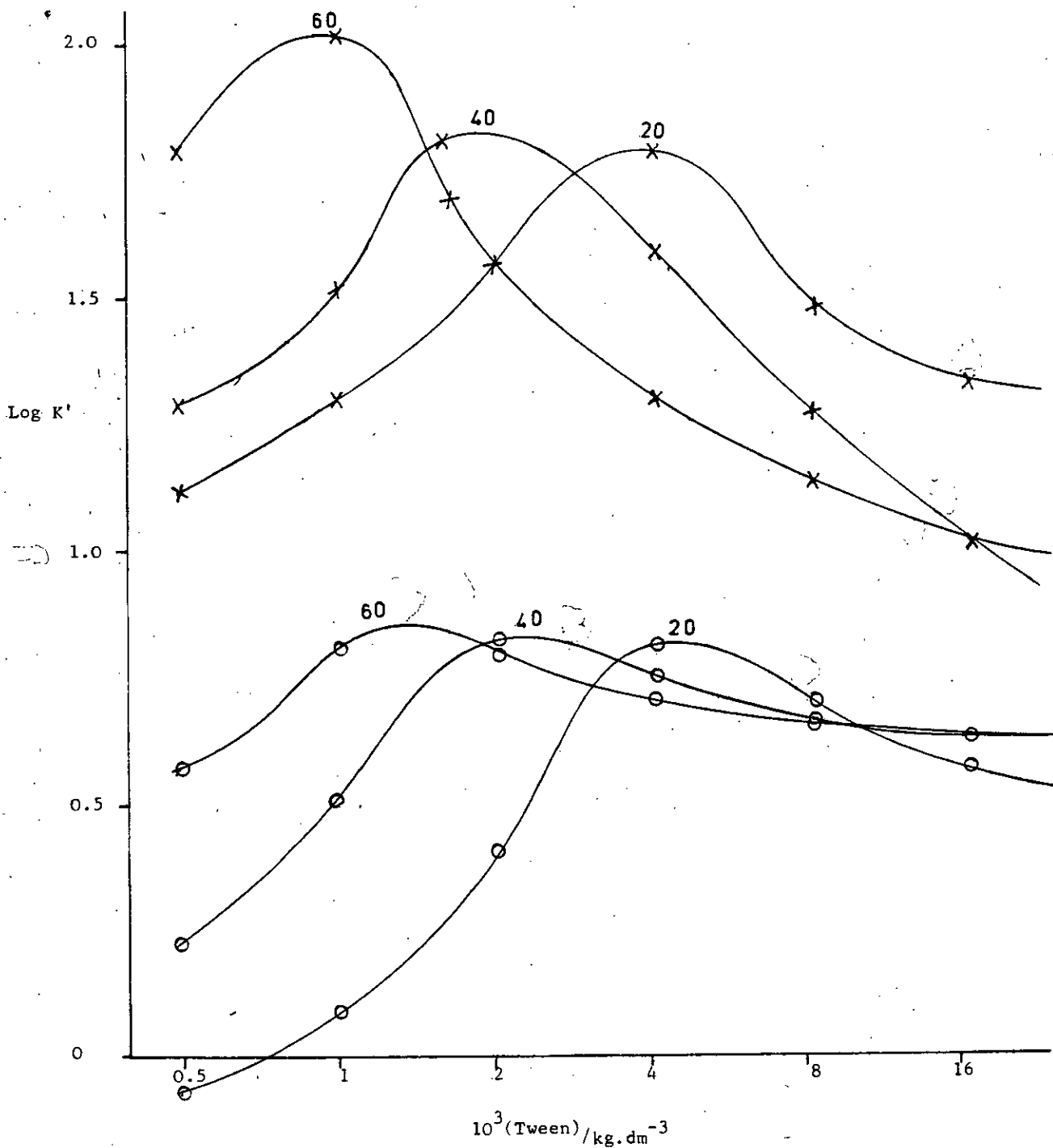$10^3$ (Tween 80)$_{kg.dm^{-3}}$

FIGURE 7-14



FIGURE 7-14

Relationship of log K' to concentration of Tweens 20, 40
and 60 in the eluent.  Conditions as in Figure 7-1.
Samples (X): Pyrene. (O) Fluorenone.

7.6    Separation of Hydrocarbons with using Tween 80 as Eluent Additive

Tween 80 has an 18 carbon alkyl chain with one element of

unsaturation.  The change in shape and hydrophobicity of the alkyl

chain associated with this unsaturation can affect selectivity and

retention behaviour, as is shown in Figure 7-13.  Retention is much

higher than with the saturated $C_{18}$ analogue and also the concentration

range of maximal retention is wider than with other Tweens, such that

it is difficult to describe a peak maximum.

7.7    General Concept of Solute Retention in the Presence of
       Different Chain Length Non-Ionic Surfactants

The composite graph, Figure 7-14 shows that the Tween

concentration at which maximum retention is observed for two eluites

(i.e. pyrene and fluorenone) is a function of surfactant hydrophobicity.

As would be expected by analogy with alkyl bonded silicas (97-99), the

largest values of K' are observed with the most hydrophobic Tween, and,

moreover, the surfactant concentration needed for maximum retention is

reduced as carbon chain length is increased.  Hemetsberger et al (99),

showed that K' was a linear function of percentage carbon content on a

series of alkyl methyl silyl "bonded phase" packings and a similar trend

is observed with these dynamically coated silica surfaces.

The similarity in elution order and relative retention of the

various analytes shown in Figure 7-9 and 7-10 implies a similarity in

separation mechanism.  As suggested in earlier studies in this series,

the result of interaction between silica and the polyol termini

of the Tween molecule is probably a modified surface bearing "brushes"

of surfactant molecules.  As the concentration of surfactant in the eluent

is increased, two competing processes occur. Firstly, coverage of the silica by "brushes" increases and, secondly, association of "bound" and free surfactant molecules (probably by hydrophobic bonding of alkyl chain) also increases.

The consequence of the first process would be an increase in retention of hydrophobic solutes with increasing brush density, but the second process would lead to an increasingly polar surface, studded with brushes with polyol termini (instead of aliphatic hydrocarbon chains). Horvath, et al (52) have shown that the elution order of solutes from columns of alkyl bonded silica packing materials is largely a function of the balance of surface and solute hydrophobicities. Accordingly, since essentially the same elution order is observed from the Tween-silica system as from octadecyl silica packing, retention in the former system should be largely due to the same properties of solute and surface.

If this hypothesis does accurately reflect the retention mechanism, then a steady increase in retentive power for chemically similar analytes should be observed with increasing brush density. This increase in retentive character will be counterbalanced by a decline in brush hydrophobicity which would ultimately lead to a decline in K' as surfactant concentration is increased. This behaviour has been observed with all single surfactant systems examined so far (c.f. Figures 7-1, 7-4, 7-11, 7-13 and 7-14 and references 101, 110, 93, 36), but may not be the case when ionic and non-ionic surfactants are used in combination (102).

## 7.8    Conclusions

Previous investigations (36,92,101) of the interaction between cationic surfactants and/or mixed non-ionic/anionic surfactants (102) dissolved in the eluent phase and LC column packings prepared from porous silica IV and zirconium IV oxides and the present data on similar interactions of non-ionic surfactants with silicon IV oxide and aluminium 111 oxides have shown that this new mode of HPLC may be used to separate a wide range of analytes varying from sulphonic acids through neutral aromatic hydrocarbons and ketones to basic amines, amino acid esters and peptides.

Selectivity in these separations may be enhanced by control of pH, solvent composition, ionic strength, chemical nature of the oxide surface, and selection of the appropriate surfactant.   In the present work, Tweens (alkyl sorbitan polyethylenoxy polyols) were shown to be strongly bonded to silica gel surfaces; indeed, they proved to be impossible to wash off the column packing with several litres of water or aqueous alcoholic solvents.  This technique of separation is rapidly and easily set up, and, many of the HPLC separations presently done by "reversed phase" chromatography on alkyl bonded silicas could be carried out with equal efficiency using this new oxide/surfactant approach.

## CHAPTER 8

### Double Surfactant Effect

8.1    Introduction

Ion Pair Reversed Phase HPLC of acidic (Dynamic Ion Exchange or Soap Chromatography) (36) and basic compounds (84) was shown in previous Chapters to proceed by a combination of at least two physico-:chemical mechanisms. That is, the retention of sample and surfactant is either through ion exchange between surfactant counter ions on surfactant attached to the surface and the desired solute, or by ion pairing between solute and surfactant in the aqueous organic eluent followed by hydrophobic interaction between the hydrophobic surface and the paired ion (S). In this respect, it would appear that interaction with dynamically coated stationary phases produced from single surfactant systems would allow separations of non ionic and ionic analytes carrying electrical charge opposite to that of the ionic surfactant. In other words, separations of amines, amino acids or other cationic species would not be favoured on silica surfaces (or any acidic surface) with a single (cationic) surfactant system. We have shown this effect in previous Chapters (Chapters 5 and 6) (101,110).

Introduction of non ionic surfactants such as laurylpolyoxyethylene glycols ("Brij") and palmityl sorbitan polyoxyethylene polyols ("Tweens" Appendix 3) (112) showed that these materials interact with both acidic gel materials such as silicon 1V and zirconium 1V oxides and the amphoteric aluminium 111 oxide (Figure 7-5) in aqueous methanolic solution to generate a hydrophobic surface. The result of this interaction

is a dynamically hydrophobized surface which may be used in ways similar to chemically bonded surfaces.

Demonstration of the usefulness of non ionic-oxide gel systems with excellent separation potential for analysis of aromatic hydrocarbons opened yet further possibilities. These were based on the hypothesis that, although anionic detergents are not bound from aqueous solutions to acidic gel surfaces, they should interact with surface-bound non ionic detergent to generate a mixed surfactant anionic hydrophobic surface. Conformation of this hypothesis was demonstrated in a paper by Wall (102) on separation of amino acids and short peptides.

In this present Chapter the effects of the addition of either cationic or anionic surfactants to silica modified by reaction with a non ionic surfactant (Tween 40) was investigated. The effects of variation of concentration of ionic surfactants at constant non ionic surfactant concentration and those of variation of non ionic surfactant concentration at constant ionic surfactant concentration were examined. The mechanism of retention is briefly explained.

## 8.2    Reagents and Solvents

Solvents and surfactants were obtained from different commercial sources as indicated in previous Chapters. Dodecyl amine (DDA) was purchased from Sigma, London. Aromatic hydrocarbon analytes indicated in Appendix 1 and amines were provided from standard commercial sources. The mixed surfactant buffer salt-organic modifier eluent systems were quite stable over the temperature range from 15 to

$60^{\circ}$C, but temperatures outside this range occasionally induced
cloudiness or even precipitation, which drastically affected the
viscous resistance of the column system. Although the solvent
was filtered through filter paper after degassing by boiling under
reflux, this filtration did not prevent a slow accumulation of
microparticulate contaminants at the top of the column which gradually
reduced column performance. It was estimated that so-called "microdirt"
reduced column life to ca 50 (working) hours. This effect can be
eliminated by careful removal of the top 2-3mm of the silica bed
and its replacement by firm tamping of a thick slurry of fresh packing
in the eluent.

For comparison purposes the ratio of water to methanol in
the eluent was kept constant at 1:1 ($v/_v$) as in the previous
investigations. Surfactants and buffer constituents were dissolved
in the aqueous portion of these mixtures before dilution with the
organic modifier, and then the pH of the eluent system was adjusted
by addition of small amounts of phosphoric acid (3M in water). The
eluites were dissolved in methanol:water (1:1 $v/_v$) before injection.

## 8.3    Proposed Retention Mechanism

The mechanism of separation using dynamically coated stationary
phases from double surfactants is apparently dominated by ion exchange
processes. Wall (102) showed that pH plays an important role in the
separation of amines, peptides and amino acids. This author also
pointed out that if the primary mechanism is ion exchange, then the
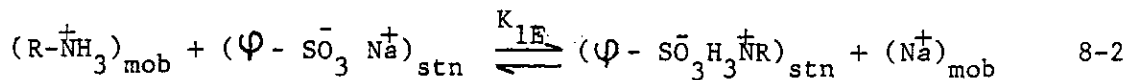relationship between K' and the reciprocal of the counter cation

concentration should be linear, and indeed this type of relation was observed in all experiments.

If it is assumed that analyte R-NH$_2$ was injected into the column, the environments used would ionise the amine as below:-

$$(R-NH_2)_{mob} + (H^+)_{mob} \xrightleftharpoons{K_{eq}} (R-NH_3^+)_{mob} \qquad \text{8-1}$$
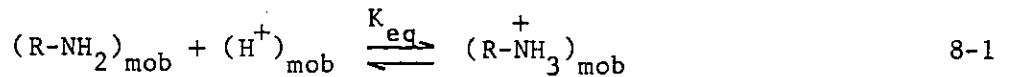
Equation 8-1 shows that amine ionisation takes place in acidic eluents. These current experiments show that (102) as the pH rises from 2 to 6, a drastic decrease in capacity ratio can be seen.

However, protonated amine can exchange with the counter cation on the surface:-

$$(R-\overset{+}{N}H_3)_{mob} + (\varphi - \bar{S}O_3\ \overset{+}{Na})_{stn} \xrightleftharpoons{K_{IE}} (\varphi - \bar{S}O_3H_3\overset{+}{N}R)_{stn} + (\overset{+}{Na})_{mob} \qquad \text{8-2}$$

So

$$K_{IE} = \frac{(\varphi - \bar{S}O_3H_3\overset{+}{N}R)_{stn}\ (\overset{+}{Na})_{mob}}{(R-NH_3^+)_{mob}\ (\varphi - \bar{S}O_3\ \overset{+}{Na})_{stn}} \qquad \text{8-3}$$

Therefore

$$K \propto D_{IE} = \frac{(R-NH_3^+)_{stn}}{(R-NH_3^+)_{mob}} = \frac{K_{IE}\ (\varphi - \bar{S}O_3Na)_{stn}}{(\overset{+}{Na})_{mob}} \qquad \text{8-4}$$
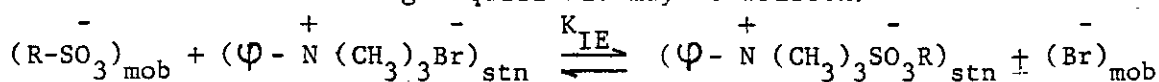
Where $(\varphi - \bar{S}O_3\overset{+}{Na})_{stn}$ is the cation exchange component of the column packing surface.

Since the concentration of cation-exchange sites $(\varphi - \bar{S}O_3)$ is fixed by the equilibrium between the (constant) eluent and the

silica packing, K' should vary inversely upon the sodium ion concentration in the eluent.

In a similar manner if separation of acidic analytes is required then an anionic exchanger is needed. Strong acids like aryl sulphonic acids are ionised at virtually all pHs accessible in HPLC usage, but weaker acids such as carboxylic acids are fully ionised only in eluents of pHs one or more units greater than their pKa values. Note that only 50% of a typical carboxylic acid will be ionised at pH ≈ 5, so retention of this analyte class will be strongly dependant on the pH of the eluent.

The anion exchange equilibria may be written:-

$$(R\text{-}SO_3^-)_{mob} + (\varphi\text{-}\overset{+}{N}(CH_3)_3Br^-)_{stn} \overset{K_{IE}}{\underset{}{\rightleftharpoons}} (\varphi\text{-}\overset{+}{N}(CH_3)_3SO_3R^-)_{stn} \pm (Br^-)_{mob}$$

$$8\text{-}5$$

Thus

$$K_{IE} = \frac{(\varphi\text{-}\overset{+}{N}(CH_3)_3\,SO_3R^-)_{stn}\,(Br^-)_{mob}}{(R\text{-}SO_3^-)_{mob}\,(\varphi\text{-}\overset{+}{N}(CH_3)_3Br^-)_{stn}} \qquad 8\text{-}6$$

and

$$K'\,D_{IE} = \frac{(\varphi\text{-}\overset{+}{N}(CH_3)_3SO_3R^-)_{stn}}{(R\text{-}SO_3^-)_{mob}} = K_{IE}\frac{(\varphi\text{-}\overset{+}{N}(CH_3)_3Br^-)_{stn}}{(Br^-)_{mob}} \qquad 8\text{-}7$$

Where $(R\text{-}SO_3H)_{mob}$ is the solute in the mobile phase and $(\varphi\text{-}\overset{+}{N}(CH_3)_3Br^-)_{stn}$ is the anion exchanger on the stationary phase surface. As in the earlier discussion on cation exchange, retention of anionic eluents will be inversely proportional to the concentration of counter <u>anion</u> in the mobile phase.

FIGURE 8-1

Possible chemical structure of dynamically generated ion exchanger, showing
interaction of silica surface, Tween 40, and SDS.
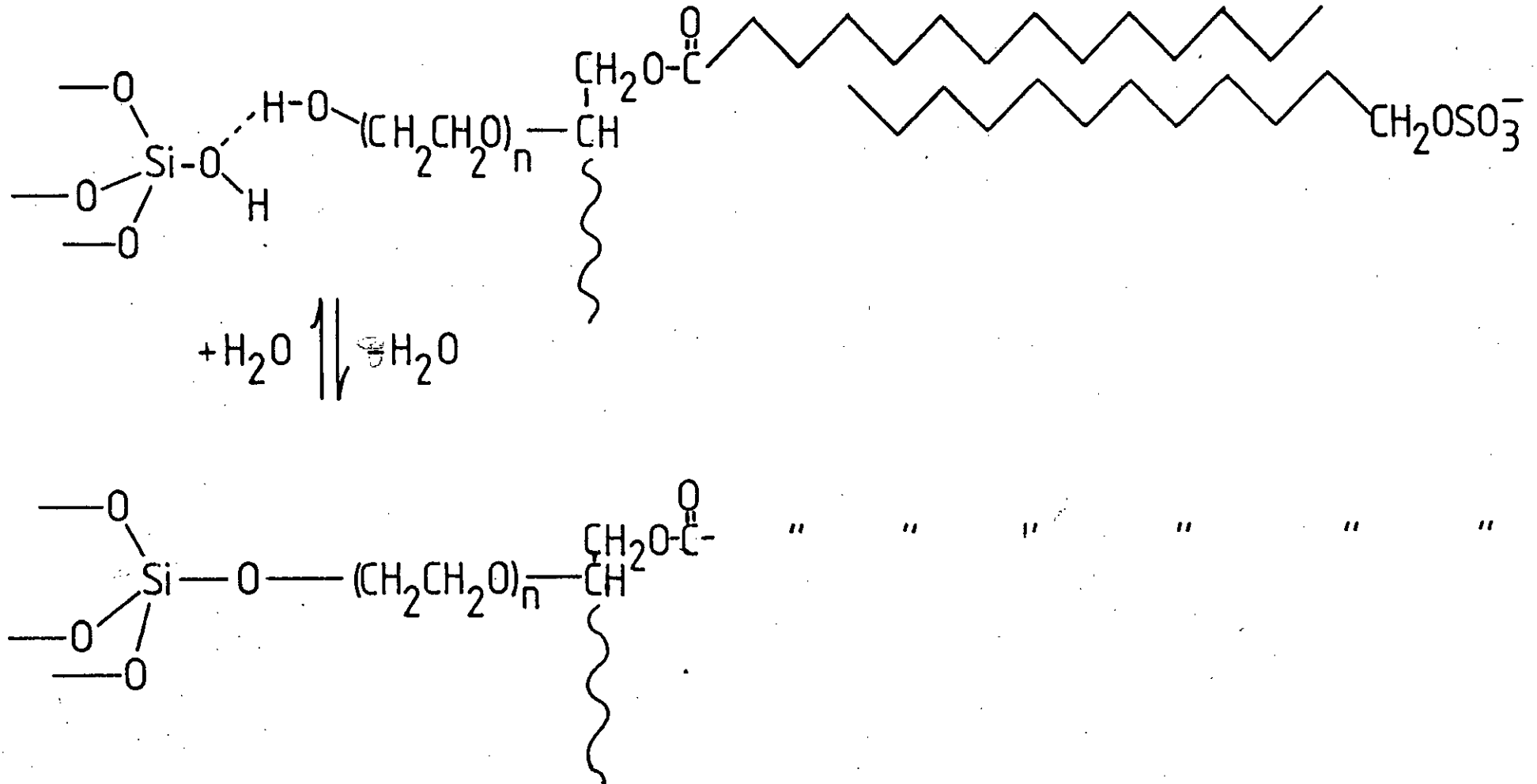
The above description of the mechanism of retention led to a wide range of investigation on the possible position and configurations of surfactant on the oxide surfaces. From discussions with colleagues and a review article by Rupprecht (48) a picture of the structure of the surface layer "seen" by eluite molecules was developed. In the first instance there is a rapid surface coverage of acidic oxides by reaction of polar groups of cationic or non ionic surfactants with gel hydroxyls and then a partial bilayer is formed more slowly by hydrophobic interaction of the long alkyl chains of the bound surfactant and those of the surfactants dissolved in the eluent. The equilibrium surface would probably be better described as resembling a synthetic ion-exchange resin, i.e. brushes bearing $-\overset{+}{N}(CH_3)_3$ or $-\overset{-}{SO_3}$ or $-OCH_2CH_2OH$ groups on the ends exposed to the mobile phase. Synthetic ion-exchange resins are well known to retain non-polar (and polar) solutes according to solute hydro-:phobicity; and indeed these results show that the described separations proceed very largely on this basis. Surface coverage of the oxide particles by the double surfactants effect is shown schematically in Figure 8-1.

8.4     Breakthrough Volume Calculations

The amount of soap (CTAB) adsorbed on the stationary phase in the presence of non ionic surfactant (Tween 40 at 2 g.1$^{-1}$) was measured by the colorimetric technique described in section 5.6.1 at two concentrations ($10^{-3}$ and $5 \times 10^{-3}$ mol.dm$^{-3}$) of CTAB.

At $10^{-3}$ mol.dm$^{-3}$ (CTAB) in the presence of 2 g.1$^{-1}$ (Tween 40)

FIGURE 8-2

Relationship of eluite retention to cationic surfactant
concentration. Eluent:methanol:water (50:50 v/v);
(Tween 40) = 2 g.l.$^{-1}$; (CTAB) = as shown.

Eluites: ⬭ = Acetophenone; ⬭ = Fluorenone; X = Naphthalene;
S = Schaffer's acid; D = Dioxy-J-acid; A = Di-J-acid;
J = J-acid   Detection at 254nm; Flow Rate = 1 ml.min$^{-1}$.

$10^4$ x (CTAB)/mole.dm$^{-3}$
+ 2 x $10^{-3}$Kg.dm$^{-3}$ (Tween 40)
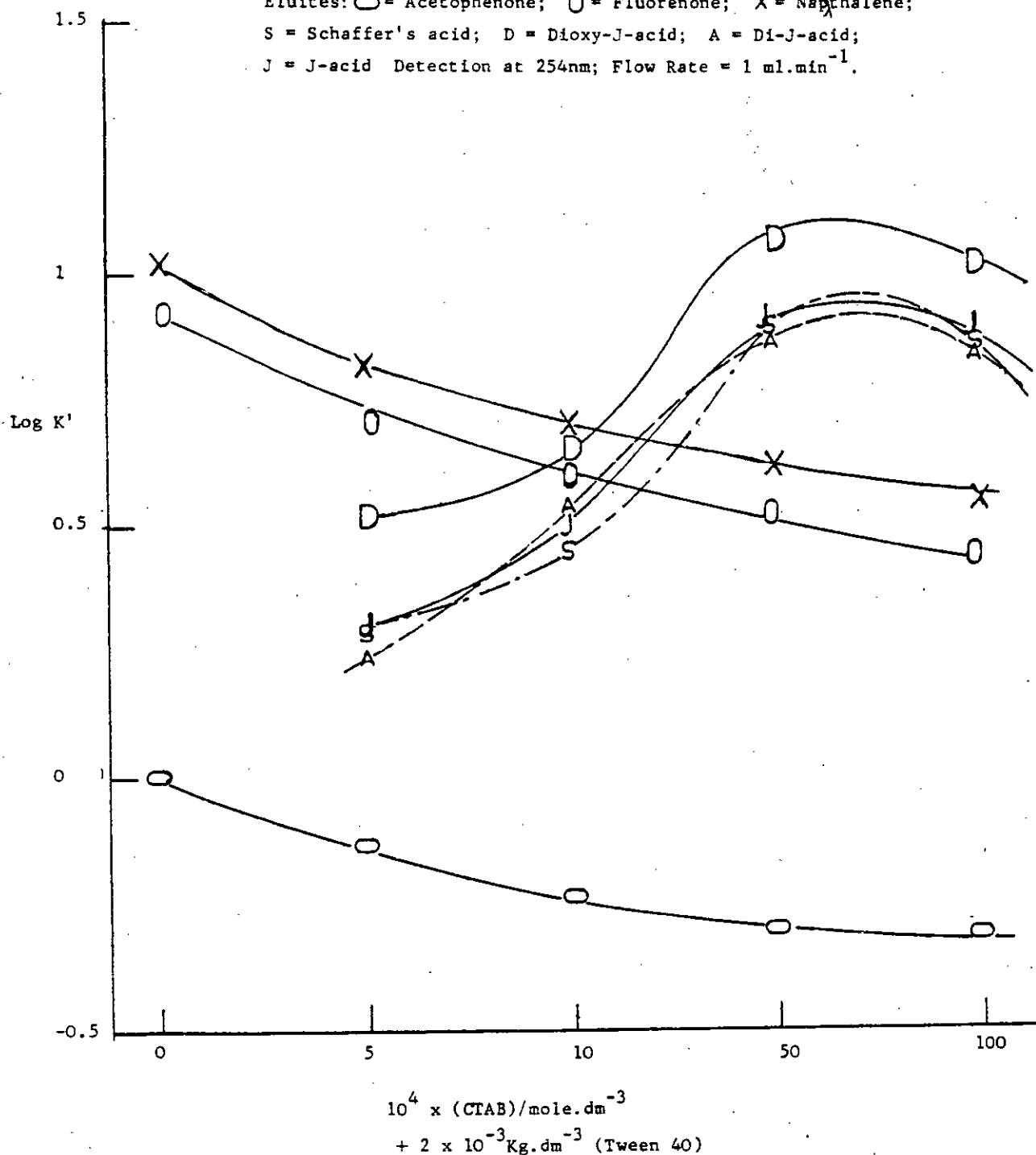
in methanol:water $(1:1 \text{ }v/_v)$ it was found that only 3.8 mg of (CTAB) were adsorbed on 1 g of silica which infers an area coverage of $7.2 \times 10^{-2}$ $\mu mol.m^{-2}$ of silica. This is roughly $1/_{20}$th of the adsorption of CTAB in the absence of Tween 40, and is comparable to cationic surfactant uptake on alkyl bonded silicas (86). This experiment as well as those results that follow in this section, demonstrate that the silica surface in equilibrium with the solution of Tween 40 and CTAB has properties arising essentially from interactions with Tween alone.

Subsequent increase in CTAB concentration of the eluent in contact with the column packed with silica which has already been equilibrated with lower CTAB concentrations shows a further increase in surface adsorption. In other words, increasing the concentration of cationic surfactant to $5 \times 10^{-3} mol.dm^{-3}$ in presence of $2$ $g.1^{-1}$ of Tween 40 and equilibrating a new column already at equilibrium with $10^{-3} mol.dm^{-3}$ (CTAB) showed a further increase of 6.4 mg on 1 g. of silica surface. At this concentration of CTAB it was calculated that there were 0.15 $\mu mol$. soap adsorbed on $1 \text{ }m^2$ of the silica surface.

## 8.5    Cationic-Nonionic Surfactant Effect on Silica Gel

As described in section 8.2, all experiments were carried out in a methanol:water $(1:1 \text{ }v/_v)$ eluent base. Figure 8-2 shows the relationship of (CTAB) to log K' at constant (Tween 40) $(2 \text{ }g.1^{-1})$. This figure postulates a clear description of the effect of double surfactant, i.e. it is shown that as the concentration of CTAB increases, surface hydrophobicity decreases due to increasing surface

FIGURE 8-3

Relationship of eluite retention to cationic surfactant
concentration. Eluent and conditions as Figure 8-2
except cationic surfactant: dodecylamine

Eluites: O = 3-phenylbutanoic acid; ◯ = 1-phenylcyclopropane-1-
carboxylic acid;

⬭ = (1-naphthyl) acetic acid;
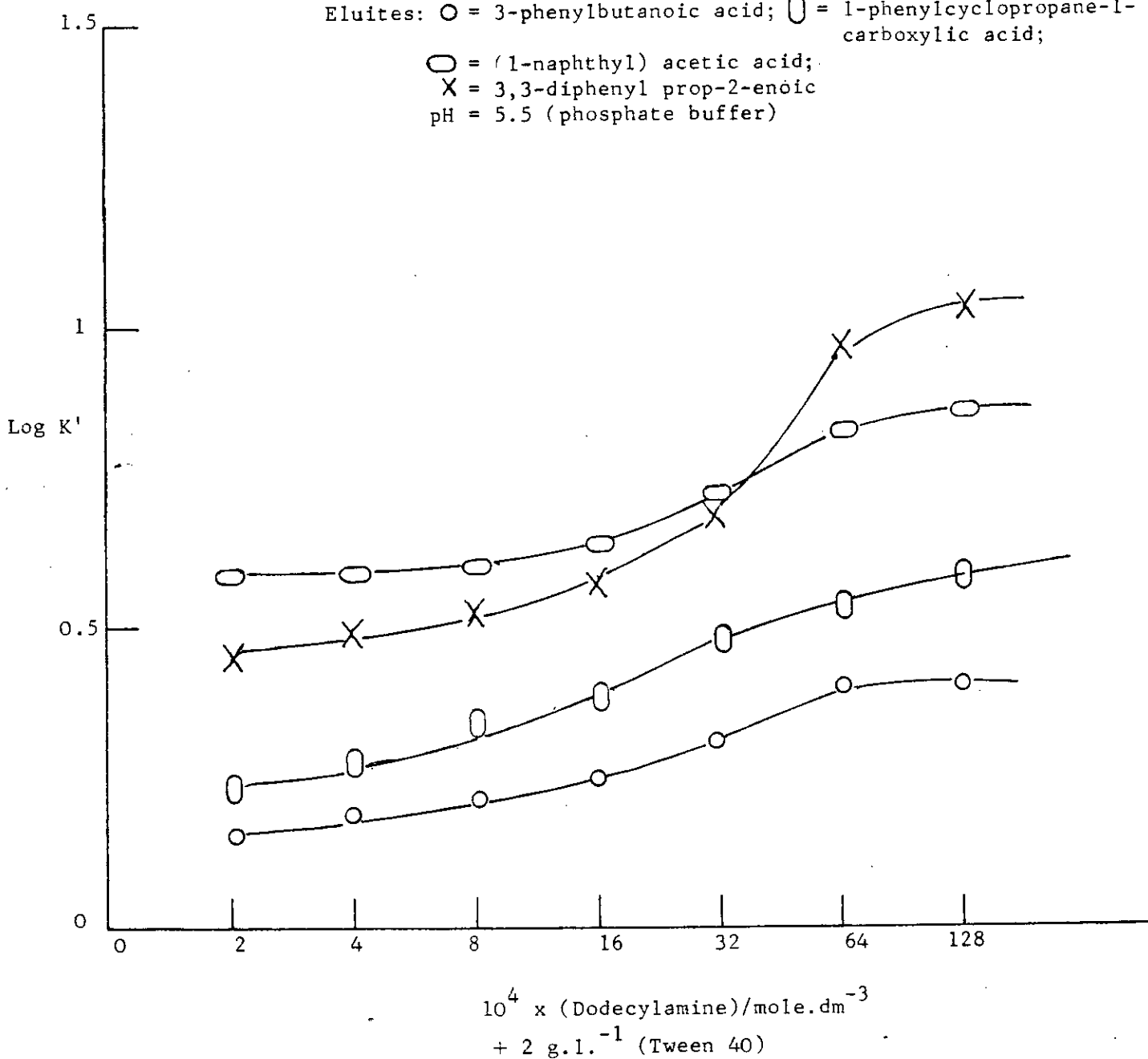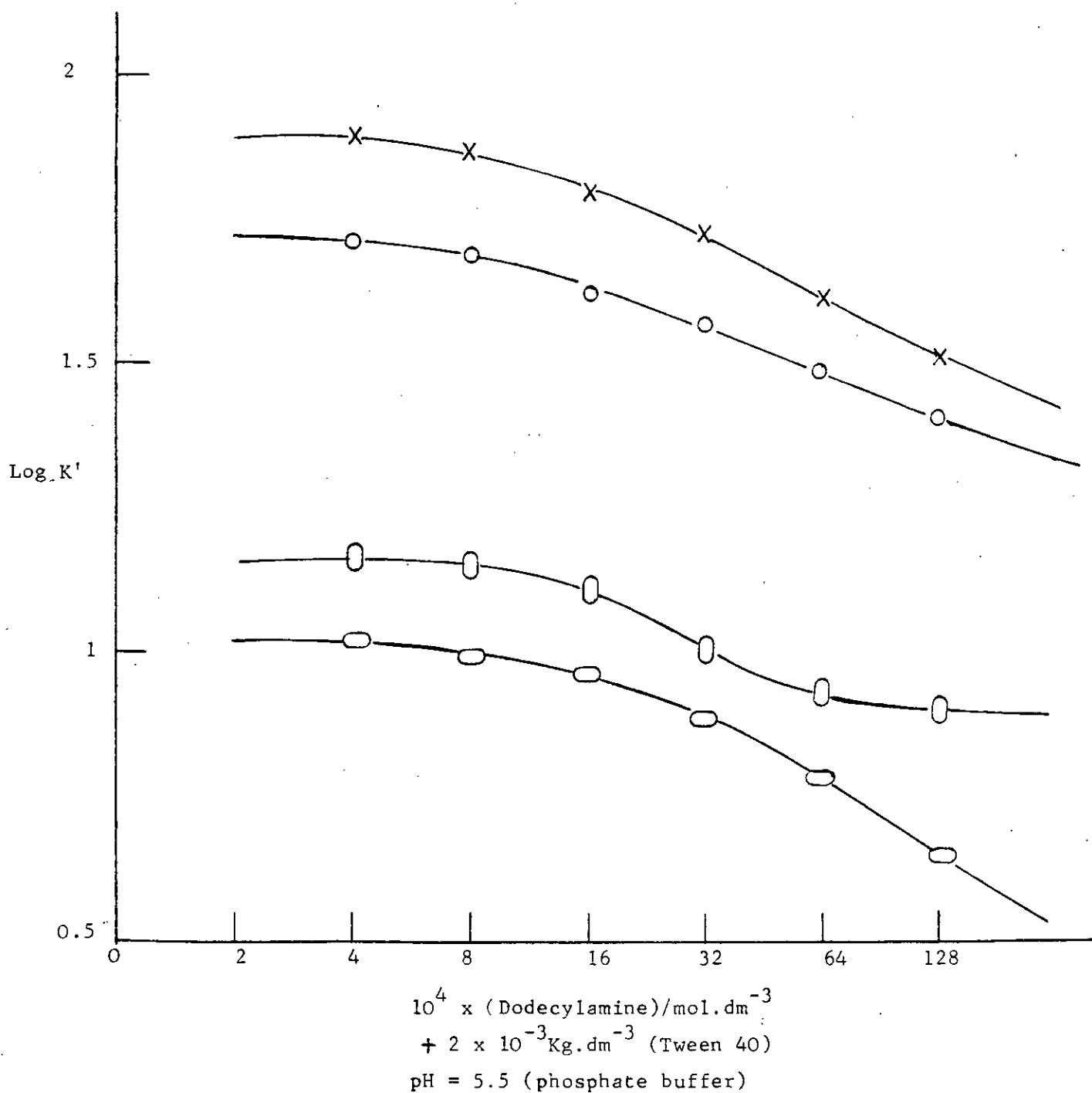X = 3,3-diphenyl prop-2-enoic
pH = 5.5 (phosphate buffer)

Log K'

$10^4$ x (Dodecylamine)/mole.dm$^{-3}$
+ 2 g.1.$^{-1}$ (Tween 40)

FIGURE 8-4

---

Relationship of aromatic hydrocarbon retention
to cationic surfactant concentration. Eluent
and conditions as Figure 8-3.

Eluites: ⬭ = Fluorenone; ◗ = Naphthalene;
○ = Anthracene; X = Pyrene

$10^4$ x (Dodecylamine)/mol.dm$^{-3}$

+ 2 x $10^{-3}$Kg.dm$^{-3}$ (Tween 40)

pH = 5.5 (phosphate buffer)

polarity from N-R$_4^+$ groups of the bound CTAB. Thus, retention of aromatic hydrocarbons decreases. But, on the other hand, increase in retention of sulphonic acids was observed due to increase in polar characteristics of the surface. It is shown that the maximum retention of sulphonic acids was achieved at $5 \times 10^{-3} \text{mol.dm}^{-3}$ of CTAB concentration.

Comparison of Figure 5-7 and Figure 8-2 shows that the maximum retention of analytes in the single surfactant mode (Figure 5-7) is slightly greater than in the mixed surfactant mode (Figure 8-2). For example, the maximum retention of Schaffer's acid ($K' \simeq 13$) at $3.2 \times 10^{-2} \text{mol.dm}^{-3}$ in the silica/CTAB system compared to maximum capacity ratio $\simeq 8.7$ in the latter technique. However, the major difference between the systems is the cationic surfactant concentration range of maximum retention. The earlier technique (single surfactant effect) showed a retention maximum at $3.2 \times 10^{-2} \text{mol.dm}^{-3}$ (CTAB) but in the newer system maximum K' was observed at a much lower concentration of CTAB ($\simeq 5 \times 10^{-3} \text{mol.dm}^{-3}$). It is clearly demonstrated that the effect of Tween on the adsorption of CTAB (and retention of analytes) is very important.

Similar results to Figure 8-2 are shown in Figures 8-3 and 8-4, where the cationic surfactant CTAB was replaced by dodecylamine (DDA) and some model carboxylic acid eluites were separated in the presence of 2 g.l$^{-1}$ (Tween 40). The problem with surface active amines is that the pH must be controlled to achieve 100% ionisation especially when weak acids (such as carboxylic acids) are to be separated.

Figure 8-3 shows the relationship of log K' to DDA concentration

on the column of silica which was pre-equilibrated with 2 g.l$^{-1}$

(Tween 40). The solvent was buffered at pH 5.5 by the addition

of a few drops of phosphoric acid (3M) during these experiments.

Maximum retention of carboxylic acids was achieved at

(DDA) = 1.3 x 10$^{-2}$mol.dm$^{-3}$in this technique. If this result is

compared to those from experiments using 5 x 10$^{-3}$mol.dm$^{-3}$(CTAB)

(Figure 8-2), it clearly shows that the longer chain, quaternary

ammonium salt is more effective than DDA although this comparison

is not strictly valid since the analytes in these two systems are

different. A further difference between retention in DDA systems

as opposed to CTAB systems can be seen from Figure 8-4, where very

slight changes in hydrocarbons retention was observed in respect

to variation of DDA concentration.

## 8.6    Anionic - Nonionic Surfactant Effects

The application of this technique can be seen in sample

separations of amines, amino acids and short peptides on silicon IV

oxide gel.

Earlier studies showed that separation of amines or (in general)

cationic species was not possible by the dynamic coating of acidic

surfaces such as silica gel with anionic surfactants. We have seen

in Chapter 6 (110) that cationic species may be separated on the

amphoteric surface of aluminium III oxides which exhibit basic

characteristics, but the selectivity and sensitivity of separation

with the alumina column packings (dp$\simeq$ 10-13$\mu$m) was poorer than those

obtained with silica (dp$\simeq$ 5$\mu$m). A possible alternative to anionic

FIGURE 8-5
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Relationship of eluite retention to anionic
surfactant concentration.

Eluent: pH 3.5; methanol:water (1:1 $v/_v$);

$(Tween\ 40) = 2 \times 10^{-3} Kg.dm^{-3}$;

(SDS) = as shown.

Eluites:  $\bigcirc$ = DL-$\alpha$ - methylbenzylamine;

$\bigcirc$ = Fluorenone;

X = Naphthalene;

$\ominus$ = DL$\alpha$ (1-naphthyl) Ethyl Amine



$10^4$ (SDS)/mol.dm$^{-3}$
+ 2 × 10$^{-3}$Kg.dm$^{-3}$ (Tween 40)
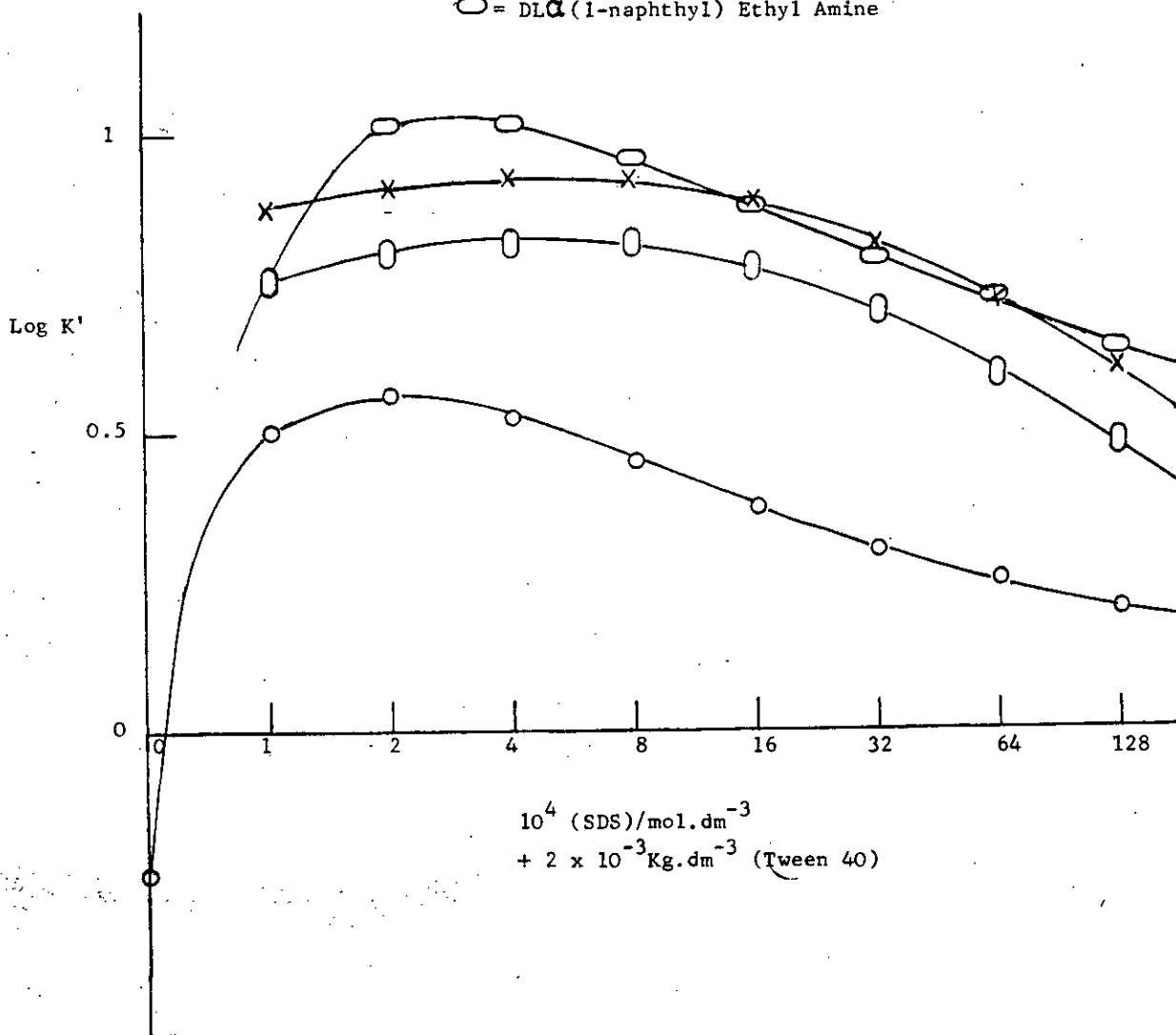
FIGURE   8-6

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Relationship of retention of four amines to concentration of anionic surfactant.  Eluent and conditions as in Figure 8-5 except anionic surfactant T.7.

Eluites:  ◖ = O-nitroaniline;    X = DL methylbenzylamine;
          ○ = P-chloroaniline;  ⬭ = DL   (1-naphthyl) Et.Amine

Log K'

1

0.5

0

-0.5

0   1   2   4   8   16   32   64   128

$10^4$ x (Tergitol 7)/mol.dm$^{-3}$
+ 2 x $10^{-3}$Kg.dm$^{-3}$ (Tween 40)
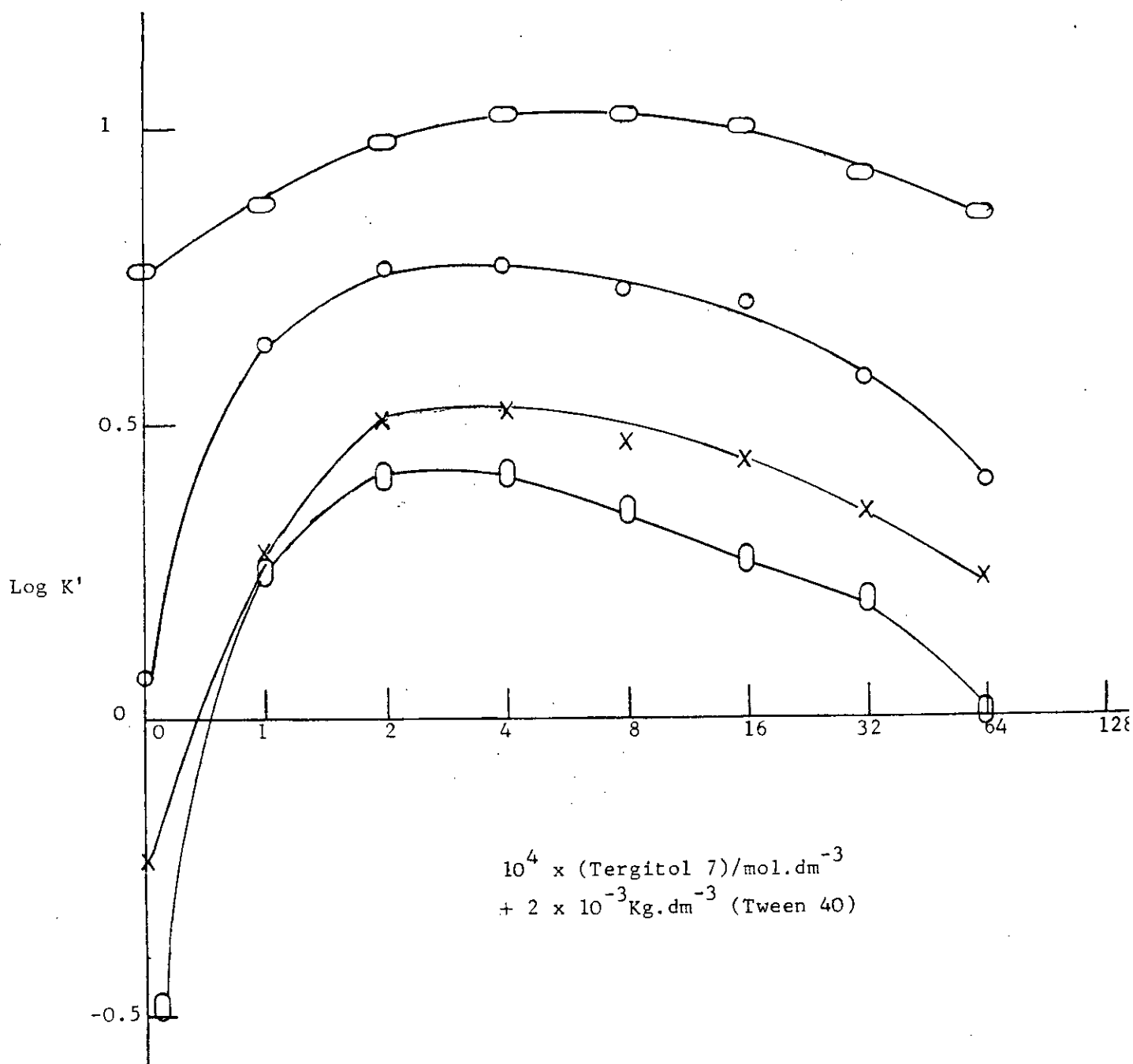
FIGURE 8-7

Relationship of four hydrocarbons retention
to concentration of anionic surfactant.
Eluent, surfactants, conditions as Figure 8-6.

Eluites:  ⬭ = Fluorenone;
          ⬭ = Naphthalene;
          ○ = Anthracene;
          X = Pyrene



$$10^4 \times (\text{Tergitol } 7)/\text{mol.dm}^{-3}$$
$$+ \; 2 \times 10^{-3}\text{Kg.dm}^{-3} \; (\text{Tween } 40)$$
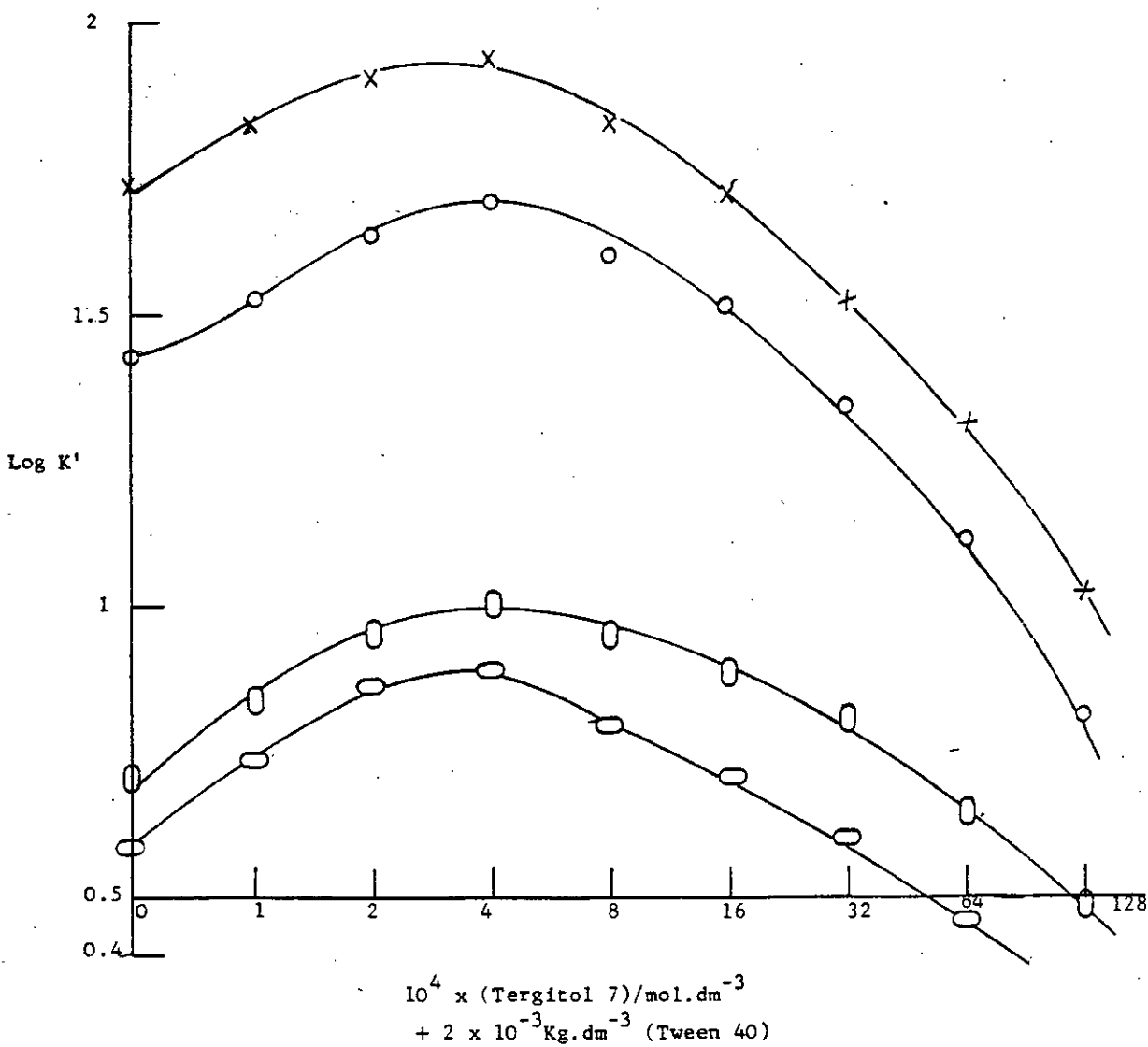
surfactant/alumina systems was a mixed non ionic-anionic surfactant modified silica column packing.

Figure 8-5 demonstrates the effect of variation of concentration of Sodium Dodecyl Sulphate (SDS) dissolved in 1:1 ($v/_v$) aqueous methanolic Tween 40 ($2g.1^{-1}$) on capacity ratio. Similarly to the separation of anionic species it was found that at pH 3.5 (phosphate buffer) capacity ratios of amines show a sharp rise until the concentration of SDS reached $2 \times 10^{-4}mol.dm^{-3}$, then a decrease in retention was observed. However capacity ratios of aromatic hydrocarbons were constant over the range from $1 \times 10^{-4}$ to $8 \times 10^{-4}mol.dm^{-3}$, then a sharp decrease in retention was observed.

This experiment was repeated with Tergitol 7 (T.7). Selectivity of this separation system appeared to be slightly different from that found in cationic surfactant experiments. Figures 8-6 and 8-7 show the relationship of capacity ratios to the concentration of T.7 at constant ($2g.1^{-1}$) (Tween 40). Figure 8-6 showed the same retention behaviour of amines as noted in Figure 8-5 but Figure 8-7 shows that capacity ratios of aromatic hydrocarbons first slightly increase due to increase in (T.7) and then sharply decrease. This effect can be seen with the less hydrophobic anionic surfactant (SDS) but is not so pronounced as with T.7 and may be due to greater decrease of solvating power of the eluent at the low concentration of anionic surfactant which may lead to a slight increase in retention of aromatic hydrocarbons. This result does not precisely correspond with the observations of Wall (Figure 1 Ref.102).

FIGURE 8-8

Relationship of four aromatic hydrocarbon retention to concentration of non ionic surfactant.

Eluent: ph = 3.5: methanol:water (1:1 $v/_v$); (SDS) = $10^{-2}$ mol.dm$^{-3}$; (Tween 40) as shown.

Eluites = as in Figure 8-7.
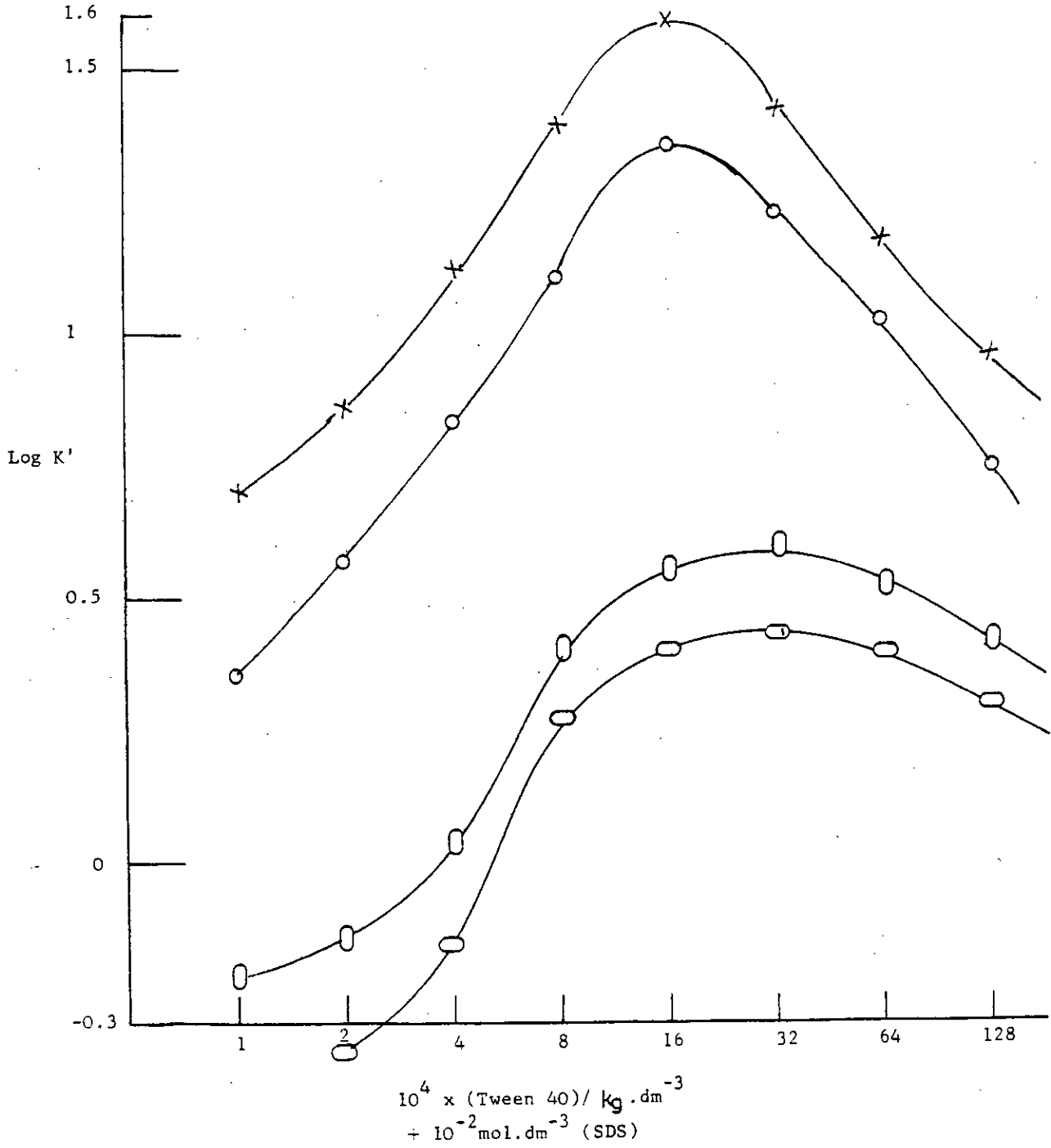
FIGURE   8-9

Relationship of eluite retention to non ionic
surfactant concentration.   Eluent, surfactants,
conditions as in Figure 8-8.

Eluites: O = O-nitroaniline;   ◐ = DL-α-Methylbenzyl Amine;
         ⬭ = P-chloroaniline;  X = DL -α-(1-naphthyl) Ethyl Amine.



$10^4$ x (Tween 40)/ kg .dm$^{-3}$
+ $10^2$mol.dm$^{-3}$(SDS)
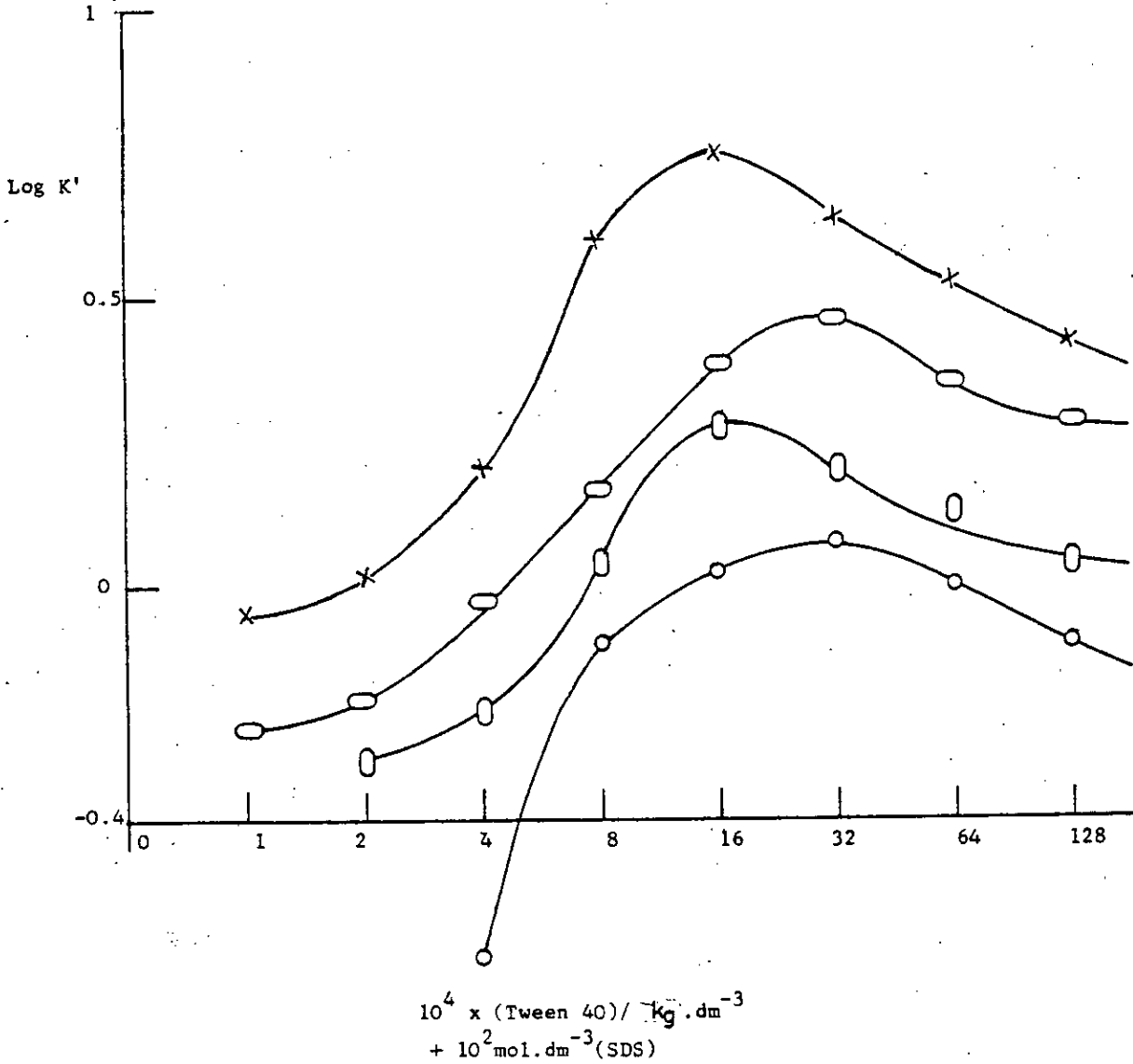
FIGURE   8-10

Separation of tyrosinyl peptides.  Eluent: pH 3.08;
40:60 (V:V) methanol:water;  (Tween 40) = 5 x $10^{-4}$ M;
(SDS) = 5 x $10^{-3}$ M; no added buffer salts.
Flow rate 0.6 $cm^3$ $min^{-1}$.  Detection at 275 nm.

(Reproduced by permission of ref.102)
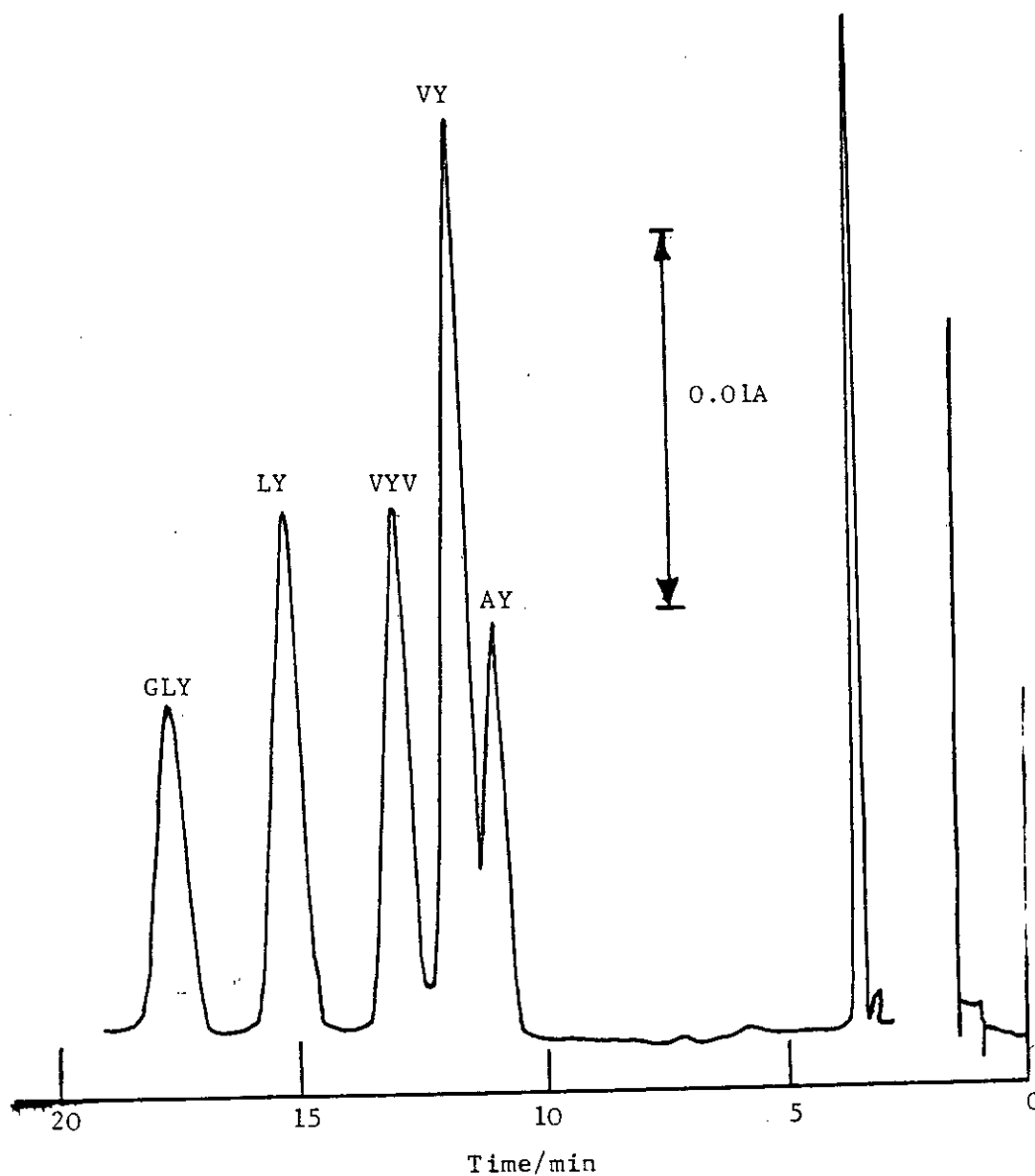


A  = Alanine
G  = Glycine
L  = Leucine
V  = Valine
Y  = Tyrosine
GLY = Glycyl-L-Leucyl-L-Tyrosine

but since solvent composition, non ionic surfactant concentration
and pH of the media are different in these two studies, they are
not necessarily directly comparable.

Figure 8-6 shows that very high retention and adequate
resolution of amines can be expected from this technique while
retention was much lower in the earlier work with the single
surfactant system (110).

Figures 8-8 and 8-9 show the relationship of capacity
ratio to concentration of non ionic surfactant at constant anionic
surfactant concentration. It is obvious that in the absence of
non ionic surfactant there is very little interaction between SDS
and the silica surface. However as soon as small amounts of
non ionic detergent were added to the solvent, retentions of amines
and aromatic hydrocarbons were drastically increased due to the
Tween interaction with the surface. All experiments in which amine
analytes were involved were carried out at the same pH (3.5).

Representative chromatogram of the separation of peptides
is shown in Figure 8-10.

8.7    Conclusions

Experimental procedures from three previous Chapters
showed that acidic surfaces such as found on silicon 1V oxides or
zirconium 1V oxides, or amphoteric surfaces such as aluminium 111
oxides can be modified dynamically simply by the addition of small
amounts of the appropriate surfactant to the contacting solvent.
The result of this addition was partial surface coverage by the

surfactant which was shown to be very difficult to remove by simple washing with surfactant-free eluents.

In this study we have shown that further alteration in surface characteristics may be achieved by the addition of cationic or anionic surfactant to an eluent/oxide column system which has been previously equilibrated with non ionic surfactant. Addition of the secondary surfactant apparently produces a surface which is complex, formed through hydrophobic interaction of the two surfactants. Indeed the second layer has not the same attachment characteristics as that from a single surfactant since it may be easily washed off and replaced by other types in the same column. We have already mentioned that selectivity can be controlled by different variables such as organic modifier concentration, ionic surfactant concentration, counter ion concentration, hydrogen ion concentration and temperature effects, so clearly yet another variable should be added to the above list. That is, control of stationary phase hydrophobicity by choice of surfactant.

The various aspects of this new mode of LC selectivity control could be an interesting subject of future research. Systematic investigation of the effects of the multiple polar functional groups of some ionic surfactants on the retention behaviour as well as separation of compounds with a wide range of multiple polar functionality is also open for future work.

## ADDITIONAL CHAPTER

### 9.1 Resolution of Enantiomers by IP-RP-HPLC

Resolution of enantiomers using chiral pairing ions either with chemically bonded stationary phases or with dynamically coated stationary phases has not yet been achieved. There are some reports on the resolution of enantiomers by HPLC. For example, Lefebvre, et al (104) synthesized an optically active macromolecular chromatographic packing from cross-linked polyacrylamide grafted with an optically active $\alpha$- amino acid. They reported that with one of these packings containing $Cu^{++}$ ions, enantiomers of valine, threonine, isoleucine, serine, phenylalanine, tyrosine, tryptophane, and asparagine are completely resolved in less than one hour. Aqueous solution of $Cu^{++}$ ion was reported as the mobile phase. In another report Nambara and his co-workers (113) resolved some amino acid enantiomers by chromatography on a silica surface covered with covalently attached chiral reagents.

The separation of ten racemic helicenes and two double helicenes was reported by Mikes, et al (114) who coated their stationary phase support with chiral $\alpha$- (2,4,5,7-tetranitro-9-fluoroenylideneaminooxy) propionic acid (TAPA). Similar results were obtained by Lochmuller and Ryall (115) who also separated 1-azo (6) helicene and heptahelicene using another type of acidic chiral derivatized surface as stationary phase and 1.5% acetonitrile in isooctane as mobile phase.

We attempted to resolve some racemic mixtures of optically

active carboxylic acids (such as 2- (and 3-) phenyl butanoic acids and 2-phenylcyclopropane-1-carboxylic acid) with dynamically coated stationary phase support. In the first experiment L-dodecyl ephedronium ion (2 x $10^{-3}$ mol.dm$^{-3}$- $10^{-1}$ mol.dm$^{-3}$) (cationic surfactant) was dissolved in methanol:water (1:1 v/$_v$). The acid analytes were injected on a column after equilibration with the surfactant and were substantially retained, but no resolution of enantiomers was found. The column/solvent system was changed to a double surfactant technique with the same methanol:water (1:1 v/$_v$) base but now containing Tween 40 (2 )g.1$^{-1}$) and varying concentrations of dodecyl ephedronium ion ($10^{-4}$- $10^{-2}$ mol.dm$^{-3}$). Retention without resolution of enantiomers was also observed in these latter experiments.

In further experiments a column was packed with SAS-Hypersil and equilibrated with an aqueous methanolic (1:1 v/$_v$) solution (5 x $10^{-3}$ mol.dm$^{-3}$) of L-camphorsulphonic acid. The pH of this solution had been adjusted with phosphate buffer to 3.5 and amine analytes (e.g. (D,L) - $\alpha$ - methyl benzyl amine, (D,L) $\alpha$- (1-naphthyl) ethyl amine, (D,L) - tyrosine, (D,L) - tryptophan methyl ester) were examined. Reasonable retentions of the amines were observed but no optical resolution was achieved at this or other concentrations of camphorsulphonic acid.

In another experiment an alumina column was packed and equilibrated with methanol:water (30:70 v/$_v$) containing L-camphor-:sulphonic acid ($10^{-2}$ to 4 x $10^{-2}$ mol.dm$^{-3}$) in the pH range from 3-4.

These last "dynamic soap" chromatographic mode separations
were as unsuccessful as the three previous methods.

The failure of these methods may be explained by
examination of their retention mechanisms. The mechanism
which was proposed in the origin of these experiments was that
if we chose a pairing ion containing a chiral centre on one
hand, and if it is assumed that an R-pairing ion is used, then
R-solute ion-pairs with R-pairing ion and the result is R-R-paired
ion, and then S-solute ion can pair with R-pairing ion and the
result would be S-R-paired ion. So it was hypothesized that
these two diastereomeric ion pairs be separable. But in fact
there are some other parameters that should have been taken into
account. The most important factor is that there should be more
than one bond between surfactant and solute as was already
reported by Cooke and co-workers (116), who resolved enantiomers
by the combined use of metal ions, hydrophobic chelating agents,
and chemically bonded n-alkyl stationary reversed phases. The
second consideration is that the chiral centre should be either
the polar centre of the surfactant or very near to the polar end.

9.2    General Conclusions

Undoubtedly ion pair chromatography has a place in
chromatographic techniques for resolving normally difficult
analyses of ionised solutes (for example, drugs and their
metabolites) in a variety of environments. One of the important
applications of ion pair HPLC is for direct analysis of solutes

in biological fluids, some without an extraction step (although the use of short pre-columns may be indicated (117)), and it would appear that these areas, i.e. of detection in formulations and biological fluids, will be those which gain most from use of ion pair HPLC. However, both the theoretical and experimental aspects of reversed phase ion pair HPLC (i.e. in which the pairing ion is located in the eluent) suggested that this is the preferred method for using ion pair techniques. This mode has the prime advantages that both the type and the concentration of the pairing ion can be easily changed even during an analysis, so that gradient elution can be carried out and to allow use of large injection volumes. This system does not suffer from column instability due to bleeding as with straight phase methods and peak symmetry is usually good.

From the discussion in section 4 and our experimental results, we suggest that the use of surface active ions in ion pair HPLC will be a profitable one for the chromatographic analysis of ionised molecules, especially when these molecules are situated in complex environments such as drug formulations and biological fluids. Ion pair RP-HPLC also gives rise to remarkably stable systems which have demonstrable flexibility in use, as afforded by the possible two dimensional change in their hydrophobicity and concentration. Surface active agents used as pairing ions can generally be obtained in the pure state and have the advantage of being ionised in the normal pH range of stable column use.

The novel form of dynamically coated stationary surface of oxide gels developed in our research may also be suitable for a wide range of biologically important analyses. The question then arises whether it is necessary to establish this novel mode of chromatography as a reliable technique and further if there is any advantage of improvement of technique over chemically bonded stationary phase technique. To answer these questions several considerations may be outlined here:-

1.    Selectivity and flexibility of the technique

2.    Sensitivity and efficiency

3.    Cost and ease of operation

4.    Time limitations

5.    Advantages and limitation of the technique

Establishment of a novel mode of chromatographic technique needs to have at least one improvement in the above considerations.

If the new form of dynamically coated stationary phase is compared with chemically bonded stationary phase, in the first instance it may be seen from the Figure 5-8 and Table 5-3 that selectivity is comparable with chemically bonded stationary phases and that the method is equally flexible while with approximately nine prime parameters (i.e. nature and concentration of pairing ion, support, mobile phase, stationary phase, temperature, pH, ionic strength and counter-ion concentration) being adjustable to produce optimal conditions. It has been demonstrated that small changes in these parameters can affect separations, and by combining these

changes with the high performances resulting from modern column

technology very rapid and highly selective separation will result.

However in the second approach, it can be argued that the value

of any technique is reflected in its ability to perform tasks more

comprehensively, and perhaps more efficiently than established

methods. This contribution has shown in some detail in our

practical sections the dynamically coated stationary phase

technique can help in the analysis of a variety of solutes. The

separating efficiency of a column is usually expressed either by

the height equivalent to a theoretical plate, H, or by the reduced

plate height, h. The variables which can affect H include column

temperature, pressure, flow rate, phase volume ratio, sample elution

time, mobile phase viscosity, and indeed ion pair parameters include

sample concentration and pairing ion type and concentration. However

preliminary investigations on plate height contribution on dynamically

coated stationary phase (Figure 5 ref.102) indicated that the column

efficiency is sufficient and comparable with chemically bonded

stationary phase systems. In fact this should be the subject of

future research, since optimisation of column parameters in this

mode of chromatography is necessary.

The advantage of novel techniques arises from the cost and

ease of operation, while the cost of commercially produced chemically

bonded materials is much higher than ordinary oxide gels. Surface

equilibration is very fast and it takes only about one hour to

equilibrate very high surface area particles. (This part was

discussed in detail in our practical sections).  One major

advantage of the technique over chemically bonded system is

that the surface can be re-generated after an acid wash which

was discussed in Section 5, but it is impossible to re-generate

chemically bonded stationary phase as a fresh oxide gel.  Since

surfactant is dissolved in aqueous eluent it is easy to alter

the system from one surfactant to another.  The technique at

present, suffers from the lack of various types of commercially

available surfactants for specific purposes (such as suitable

surfactant for separation of optically active materials).

Another disadvantage of the technique at present is that there

is no simple procedure for separation of surfactant from the

analyte after eluting from the column, therefore this technique

can not be readily used for preparative purposes.  This problem

also exists with ion pair chemically bonded HPLC.

9.3    Future Aspects of Dynamically Coated Stationary Phase

There are several potential areas of research in this novel

form of chromatography.  Some of them are outlined here:-

1.    Investigation on the use of different surfactant

on different oxide gel surfaces or derivatized

surfaces.

2.    Development of new kinds of surfactant.

3.    Application of the technique on the other

types of liquid chromatography (such as TLC).

4.    Application of the technique on the other

subjects (such as pharmaceutical, agriculture, biology, and so on).

5.      Investigation on the effect of different variables on the column performance.

In our experimental work we have applied some cationic, anionic, non-ionic, and double surfactants on silicon 1V oxide, zirconium 1V oxide and aluminium 111 oxide surfaces. The results indicated that although separation of model analytes on zirconia and alumina surfaces were sufficient for our purposes but some more investigation is needed, on either surface derivatisation or discovery of new oxide surfaces for better column efficiency, particularly when specific separations or use of higher pHs are required.

It was indicated in our previous chapters that there are some improvements in surfactant types with different kinds of chain length as reported by Hemetsberger, et al (97-99) and Tomlinson, et al (84,86), but more research in this line which consists of the preparation of different surfactants for general and specific purposes are required. This approach also involves the observation of the effect of different additives (organic or inorganic) on the surfactant formula and the effect of them on column performance.

Application of the technique on the other types of chromatography can be another field of future research. Armstrong and co-workers (118) have applied some cationic (CTAB) and anionic (SDS) surfactants on alumina and polyamide bonded alumina surface

using TLC plates. $R_f$ values of some drugs showed that there are potential values in the TLC technique.

Application of the technique for separation of some biologically, and/or pharmaceutically important substances is also a very important subject of research. Wall (102) has applied this technique for separating some amino acids and short peptides using double surfactants on a silica support. Wall (unpublished results) has also applied this double surfactant technique to the separation of some anions in human urine in the study of an enzyme deficiency disease.

Finally, systematic investigations on the effect of different environmental parameters is also needed. Since the effect of temperature, salt concentration, pH on chemically bonded stationary phase was reported by Kraak and Huber (35) and Tomlinson, et al (84), indicated that the effect of these parameters are critical in retention behaviour. Although preliminary investigation on the effects of pH and salt concentration were reported in our studies, there remains much more research to be done on the mechanism of the effects of variation of these parameters on solute retention as well as the effects of temperature variation on separation.

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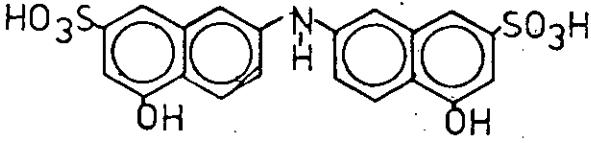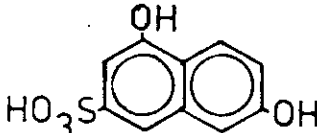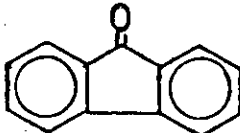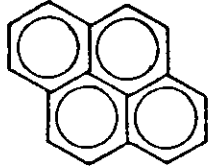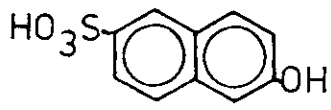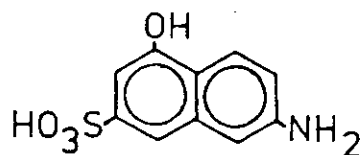
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## APPENDIX 1

Aromatic Hydrocarbons, Ketone and Sulphonic acid analytes.

## ANALYTES

| Name | Formula | Elution Order ($2.7 \times 10^{-2}$ M Cetrimide) |
|------|---------|--------------------------------------------------|
| A  Anthracene | | 3 |
| B  Di-J-acid | | |
| C  Dioxy-J-acid | | |
| D  Fluorenone | | 1 |
| E  Naphthalene | | 2 |
| F  Pyrene | | 4 |
| G  Schäffer's acid | | |
| H  J-acid | | |

## APPENDIX 2

### Physical Properties of Zirconium Oxide "S" Grade

| | |
|---|---|
| Specific Gravity | 5.7 |
| Tamped Bulk Density $(gr/cm^3)$ | 2.4 |
| Average Particle Size (micron) * | 10-15 |
| Average Particle Size (micron) ** | 6-8 |

Particle Size Distribution *

| Micron | 23 | 19 | 15.5 | 13 | 9 | 7 | 5 | 3 |
|---|---|---|---|---|---|---|---|---|
| % less than | 95 | 90 | 70 | 50 | 20 | 10 | 5 | .2 |

| | |
|---|---|
| Specific Surface Area $(m^2.gr^{-1})$ ++ | 2-4 |
| Hardness (Mohs' Scale) | 7 |

*      Sedimentation (Andreasen)

**     Fisher Sub-Sieve Sizer

++     Nitrogen Absorption

## Molecular Formula and Chemical Names of Non-Ionic Surfactants

| Commercial Name of Surfactant | Chemical Name | Formula | Molecular Weight |
|---|---|---|---|
| Tween 20 | Polyoxyethylene Sorbitan Monolaurate | $CH_2O - \overset{O}{\underset{\shortmid}{C}} - (CH_2)_{10} - CH_3$ <br> $O-CH$ <br> $CH - O - CH_2\ CH_2\ OCH_2\ CH_2\ OCH_2CH_2OCH_2CH_2OH$ <br> $CH - O - CH_2\ CH_2\ OCH_2\ CH_2\ OCH_2\ CH_2\ OH$ <br> $CH - O - CH_2\ CH_2\ OCH_2\ CH_2\ OCH_2\ CH_2\ OH$ <br> $CH_2$ | $C_{38}\ H_{74}\ O_{16}$ <br><br> 786 |
| Tween 40 | Polyoxyethylene Sorbitan Monopalmitate | $CH_2O - \overset{O}{\underset{\shortmid}{C}} - (CH_2)_{14} - CH_3$ <br> The rest of molecule as above | $C_{42}\ H_{82}\ O_{16}$ <br><br> 842 |
| Tween 60 | Polyoxyethylene Sorbitan Monostearate | $CH_2O - \overset{O}{\underset{\shortmid}{C}} \ (CH_2)_{16} - CH_3$ <br> The rest of molecule as above | $C_{44}\ H_{86}\ O_{16}$ <br><br> 870 |
| Tween 80 | Polyoxyethylene Sorbitan Monooleate | $CH_2O - \overset{O}{\underset{\shortmid}{C}} - (CH_2)_7 - CH = CH\ (CH_2)_7 - CH_3$ <br> The rest of molecule as above | 868 |

# HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

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## SUMMARY

A novel form of hydrophobic ("reversed-phase") high-performance liquid chromatography is described. Column packings are conventional porous oxide (silica and zirconia) gels with relatively hydrophilic surfaces whose polarity is drastically altered by reaction with quaternary ammonium cationic surfactant dissolved in the eluting solvent. Retention and separation of mixtures of aromatic hydrocarbons, an aromatic ketone and aryl sulphonic acids is shown and is tabulated as a function of mixed solvent (methanol–water) composition and also as a function of surfactant concentration.

Column resolving power in this mode of separation is unchanged from that obtained with the same column packings used in the adsorbent mode with non-polar solvents.

## INTRODUCTION

The usage of "reversed-phase" liquid chromatography has developed rapidly since the introduction of well-graded microparticle ($d_s \approx 5\,\mu m$) materials based on the virtually complete coverage of the reactive silanol groups of silica gels by covalently attached hydrocarbon chains[1,2]. Many authors have pointed out the potential of a high-performance liquid chromatographic (HPLC) mode which may encompass virtually all types of analyte from the hydrophobic polycyclic aromatic hydrocarbons to such polar molecules as amino acids and sulphonic acids. This versatility is achieved by the control over selectivity inherent in partially aqueous solvent systems with which a wide variation in hydrophobicity (content of organic modifiers, *e.g.*, methanol), pH and ionic strength may be easily accommodated.

Separations of the most polar analytes are usually achieved by some variation of the ion-pairing techniques originated by Horváth and Lipsky[3] and Eksborg and Schill[4], in which a relatively low concentration of a charged solute (*e.g.*, alkyl sulphonic acid or alkyl quaternary ammonium salt) in the solvent allows formation of hydrophobic ion pairs with the analyte. Hydrophobic interaction of these ion pairs with the covalently bonded alkyl chains on the packing surface then provides the mechanism for separation of the polar analytes.

In some experiments which preceded this current study Gilbert and Wall[5] found that porous ceria column packings gave retention data similar to those reported by Knox and Laird[6] in their studies of "soap" chromatography on short-chain alkylated silica. The conclusion of this earlier study[5] was that "soap" chromatography provided a useful probe of the relative hydrophobicity of the surface of the column packing. Subsequently, Horváth et al.[7] demonstrated that the diagnostic sample retention–surfactant concentration relationship is not necessarily a function solely of the column-packing surface hydrophobicity but also (and primarily) is derived from the hydrophobic character of the added surfactant.

Accordingly, at the beginning of this present work in which a porous zirconia gel was tested as an HPLC column packing, the interaction of conventional porous silica gel packing with quaternary ammonium salt surfactants was re-examined. It soon became obvious that the dissolved surfactant reacts with native oxide surfaces to produce a new packing–solvent interface which behaves very much as do "bonded phase" column packings. The properties of this "solvent-generated" hydrophobic surface were used for HPLC separations of a number of non-polar and polar analytes. Apparently, many analyses which have been done on surface-modified column packings could have been accomplished with equal efficiency using conventional oxide gel packing materials without prior chemical modification.
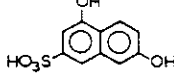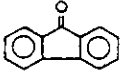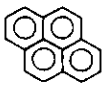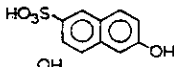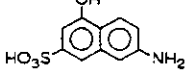
## EXPERIMENTAL
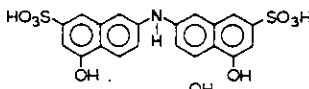
The chromatographic measurements were carried out on a variety of equipment, including Altex Model 110 and Waters M6000A pumps, Cecil Model 212 and Applied Chromatography Systems Model 710 ultraviolet flow photometric detectors and SGE microsyringes in association with laboratory-constructed septum injector–column systems.

Two silicaceous column packing materials were used: Hypersil (Shandon, London, Great Britain), a spherical silica gel, $d_p = 5\,\mu$m, surface area $\approx 200\ \mathrm{m^2\,g^{-1}}$; and ODS (octadecylsilyl)-Hypersil (Shandon), in which the adsorbent activity due to residual silanol groups has been reduced to a minimum. The zirconium oxide gel was very kindly supplied by Magnesium Elektron (Manchester, Great Britain) and was their highest purity, spherical microparticulate $S$ grade. This zirconia was modified by hydrothermal treatment to increase effective pore size and had a surface area of 9 $\mathrm{m^2\,g^{-1}}$; a fraction ($d_p = 10 \pm 3\,\mu$m) was isolated by hydraulic elutriation and was used in subsequent experiments.

Columns were made from Apollo grade (Accles and Pollock, Oldbury Warley, Great Britain) polished (4.6 mm diameter) bore AISI 316 grade stainless-steel tubing in lengths of 100 or 125 mm. They were packed by the "upward-flow" slurry-packing technique described by Bristow[8] at ca. 300 bar liquid pressure; both silica and zirconia were suspended and packed in methanol.

The sulphonic acid solutes were kindly gifted by I.C.I. Dyestuffs Division (Macclesfield, Great Britain) and are listed under their common names and structural formulae in Table I. Hydrocarbon and ketonic solutes are also listed in Table I and were obtained from the usual commercial sources. Purity of the methanol solvent used in these experiments proved to be critical for reproducible measurements; attempts to use reagent grade methanol from several suppliers gave

TABLE I

ANALYTES

| Name | Formula | Elution order (2.7 × 10⁻² M cetrimide) |
|------|---------|----------------------------------------|
| A Anthracene | | 3 |
| B Di-J-acid | | |
| C Dioxy-J-acid | | |
| D Fluorenone | | 1 |
| E Naphthalene | | 2 |
| F Pyrene | | |
| G Schäffer's acid | | |
| H J-acid | | |

variations in solute retention over a four-fold range. Consistent solvent action was obtained from AR grade methanol after distillation from magnesium methoxide solution or by use of HPLC grade methanol obtained from Rathburn Chemicals (Walkerburn, Great Britain).

Hexadecyltrimethylammonium bromide, puriss ("cetrimide"), was purchased from Fluka (Buchs, Switzerland).

RESULTS AND DISCUSSION

*Separations on silica gel*

An equal volume (1:1) mixture of methanol and water was used as eluent in all measurements quoted in Fig. 1, with the addition of sufficient cetrimide to cover the concentration range from $1.7 \times 10^{-3}$ to $5.5 \times 10^{-2}$ $M$. All the data used to develop the relationship between log $k'$ and cetrimide concentration were collected on columns which had previously been exposed only to solvent containing the measured or lower concentrations of the same added surfactant. This precaution was thought to be necessary since it was observed that attempts to convert a silica–cetrimide column to one in equilibrium with a different quaternary ammonium salt

(benzyltrimethylammonium chloride and propyltrimethylammonium iodide were used in preliminary experiments) gave retention data different from those of silica columns which had been in contact with these other salts only. Moreover, several attempts to regenerate a "clean" silica surface by acid washing (dilute acetic, formic and nitric acids were examined) to remove "bound" surfactant did not generate a surface capable of interacting to the original extent with added surfactant. Although these "acid-washed" columns did not retain their surfactant binding properties, the washing process did apparently give a silica surface similar to the original "as packed" condition, since measurements of retention of some standard solutes in the adsorption LC mode with 1% methanol in hexane as solvent were essentially identical before and after surfactant–acid treatment.
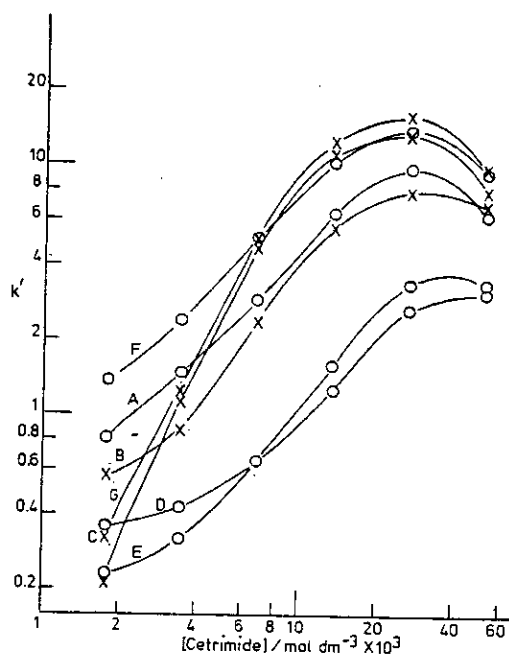


Fig. 1. Relationship of sample retention ($k'$) to concentration of added surfactant. Eluent: methanol–water (50:50, v/v). Analytes coded as in Table I. Column packing: Hypersil.

In spite of this clear evidence of a surface which could not be readily regenerated on the column packing, it was found that retention data from several columns which were equilibrated in the same way with the same surfactant were consistent. Furthermore, the retentive characteristics of fully equilibrated cetrimide–silica columns were constant over periods of several weeks.

The relationship of log $k'$ (of a range of analytes) with cetrimide concentration is shown in Fig. 1; there was no retention of these solutes on silica which had not been exposed to cetrimide with the same solvent in the absence of added surfactant. Although the shapes of the curves describing the variation of $k'$ with the cetrimide concentration are all similar, there are several changes in the order of elution of solutes evident as crossover points of the various curves. These changes in

selectivity probably reflect the differences in chemical reactivity of such a diverse collection of analytes (mono- and disulphonic acids, hydrocarbons and a ketone), and are an advantage to the practising chromatographer, since they imply that some degree of control over relative as well as absolute retention may be obtained by varying the concentration of added surfactant. This control may be extended by variation of the solvent composition as well, although changes in selectivity arising from change of water content are small compared to cetrimide-induced differences.

Our results do not precisely parallel those of Knox and Laird[6], which were obtained on silica and SAS-silica packings, in that virtually all our measured $k'$ vs. cetrimide concentration curves pass through a maximum in $k'$ at ca. $2.7 \times 10^{-2} M$ cetrimide. However, our results were obtained with silica of lower surface area ($200 \text{ m}^2 \text{ g}^{-1}$ as opposed to $500 \text{ m}^2 \text{ g}^{-1}$) and with a different solvent system. According to Horváth et al.[7] the shape of these curves is a function of a balance of interaction between the packing surface and the solvent. The 1:1 (v/v) methanol–water mixture used in these experiments is more polar than the 3:1 (v/v) propanol–water mixture used by Knox and Laird[6]. This difference in solvent polarity is reflected in the retention measurements: $k' = 0.15$ for Schäffer's acid in the propanol–water solvent and $k' = 13$ for the same solute in the methanol–water solvent when both eluents contain the same ($2.7 \times 10^{-2} M$) amount of cetrimide.

The amount of cetrimide adsorbed on the silica surface was measured by the colorimetric method of Knox and Laird[6], and the polarity difference in the solvents employed was again reflected in the coverage data. At $1.4 \times 10^{-2} M$ cetrimide it was found that 64 mg of surfactant were sorbed on 1 g of silica, which infers an area coverage of 0.5 molecules per $\text{nm}^2$ of silica surface, roughly equivalent to one sixth of the maximum possible coverage if sorption proceeds by $-\overset{+}{N}(CH_3)_3$ reaction with the surface. The highest reported[9] silica surface coverage by covalently attached $-Si$ $(CH_3)_3$ groups is ca. 2.3 groups per $\text{nm}^2$, so the solvent-generated hydrophobic surface is clearly less well covered than the best commercial "bonded-phase" packings.

Table II indicates that the effective differences between a solvent-generated hydrophobic packing and an ODS–TMS bonded-phase packing are less than might have been estimated from the surface coverage data. Analyte retention was measured with the same solvent system on both columns. Selectivity calculations ($\alpha_{1-2} = k'2/ k'1$) reinforce the apparent similarity in the chromatographic behaviour of these two different column packings.

Lack of suitable samples of alkyltrimethylammonium halides prevented a

TABLE II

COMPARATIVE RETENTION DATA ($1.4 \times 10^{-2} M$ CETRIMIDE)

| Datum | Hypersil | ODS-Hypersil | Zirconia |
|---|---|---|---|
| $k'$ Fluorenone (1) | 2.3 | 3.7 | 0.38 |
| $k'$ Naphthalene (2) | 2.7 | 5.0 | 0.63 |
| $k'$ Anthracene (3) | 10.7 | 17.3 | 2.5 |
| $k'$ Pyrene (4) | 18.5 | 28.3 | 4.1 |
| $\alpha_{1-2} = k'(2)/k'(1)$ | 1.14 | 1.37 | 1.66 |
| $\alpha_{2-3} = k'(3)/k'(2)$ | 4.00 | 3.47 | 4.00 |
| $\alpha_{3-4} = k'(4)/k'(3)$ | 1.73 | 1.63 | 1.65 |

systematic investigation of the effect of alkyl chain length on solute retention, but preliminary investigations with propyltrimethylammonium ($CH_3CH_2CH_2-\overset{+}{N}[CH_3]_3$) and benzyltrimethylammonium ($C_6H_5CH_2-\overset{+}{N}[CH_3]_3$) halides showed that $k'$ does increase with chain carbon number. Hemetsberger et al.[10] showed that $k'$ was a linear function of percentage carbon content on a series of alkylmethylsilyl "bonded-phase" packings, and if the retention mechanism is as closely related to that of the alkyl bonded phases as these results suggest, a similar dependence on $k'$ upon alkyl chain length of the added surfactant would be predicted for the solvent-generated system.

Fig. 2 is a representative chromatogram of simple mixtures using methanol–water–cetrimide solvent systems with spherical silica column packing. Fig. 3 is a chromatogram of the same mixture with the same eluent used in Fig. 2, but on the octadecyl "bonded phase" ODS-Hypersil. As suggested by the data of Table II, the two separations are very similar.
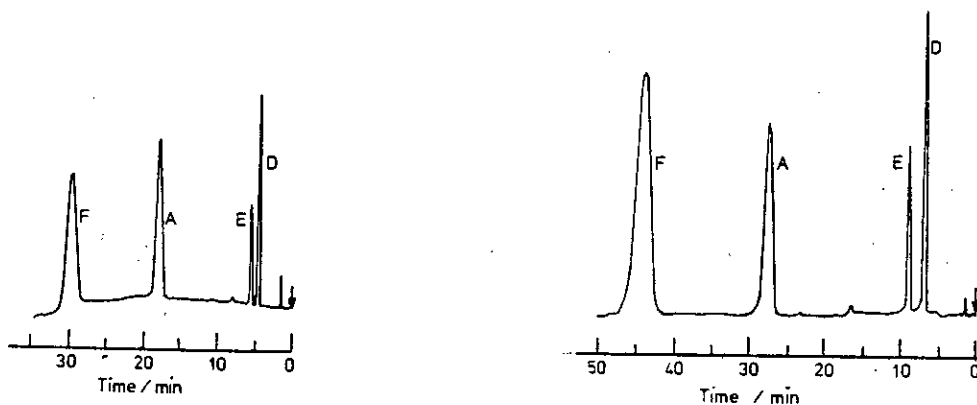


Fig. 2. Separation of four non-polar aromatic compounds (cf. Table I for code) on a column (125 × 5 mm) of Hypersil. Flow-rate: 1 cm³ min⁻¹. Eluent: methanol–water (50:50, v/v) solution of cetrimide (1.4 × 10⁻¹ mol dm⁻³).

Fig. 3. Separation of four non-polar aromatic compounds on a column 125 × 5 mm) of ODS-Hypersil. Conditions as in Fig. 2, except for the eluent: methanol–water (60:40, v/v).

### Separations on porous zirconia

The data shown in Fig. 4 were obtained on porous zirconia columns by development with the same solvent system used in the experiments which gave the information recorded in Fig. 1 and Table II. A lesser retentive power would be predicted for this zirconia (9 m² g⁻¹, calculated pore = 36 nm) than for the silica (200 m² g⁻¹, calculated pore = 12 nm) described above, but the disparity in "available" surface is not as great as it appears, since the packing surface area in a typical 125-mm long column would be about 240 m² for the silica (bulk density ca. 0.5 g cm⁻³) and 43 m² for the zirconia (bulk density ca. 4 g cm⁻³). In fact, $k'$ (max.) for pyrene was ca. 18.5 on silica (at 1.4 × 10⁻² M cetrimide) and 4.1 on zirconia (at 1.4 × 10² M cetrimide), so retention is slightly higher on the latter packing than a simple surface area ratio would suggest.

The structure of the pores in this zirconia is clearly not ideal for HPLC usage, since even a well-fractionated sample (according to particle diameter) did not
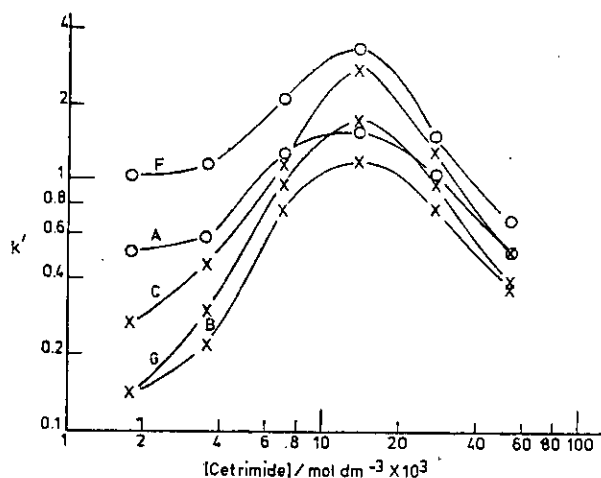
Fig. 4. Relationship of sample retention ($k'$) to concentration of cetrimide. Eluent as in Fig. 1; analyte coding as in Table I. Column packing: hydrothermally modified zirconia, $d_p \approx 10 \mu m$.

exhibit plate heights smaller than 10–12 particle diameters at optimum flow-rates as is evident from Fig. 5 and 6. However, column efficiency was adequate to the purpose and it was felt that the surface characteristics of this zirconia gel would be comparable to those of a gel with the smaller pores (pore $\approx$ 6–12 nm) characteristic of high-efficiency LC packings.
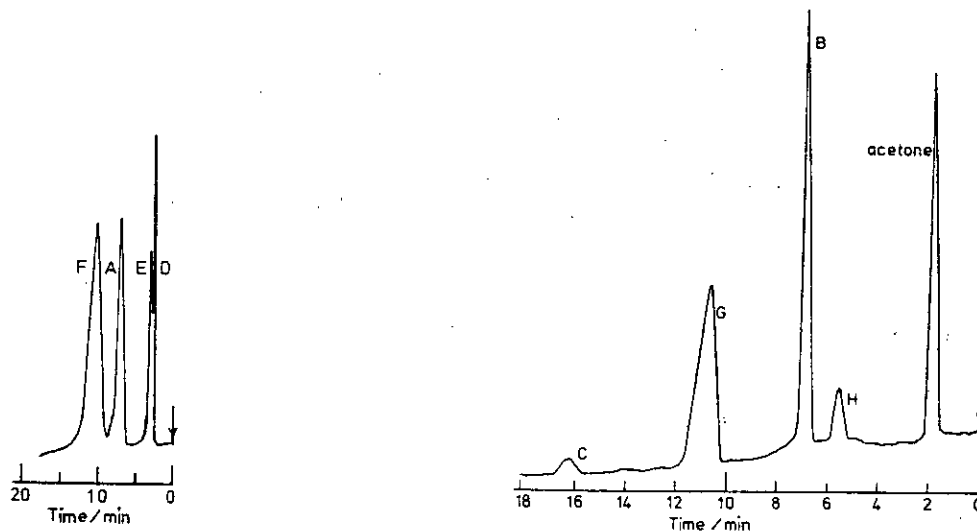


Fig. 5. Separation of four non-polar aromatic compounds as in Fig. 2 and 3. Operating conditions as in Fig. 2 except flow-rate = 0.6 cm³ min⁻¹. Column packing: hydrothermally modified zirconia, $d_p \approx 10 \mu m$.

Fig. 6. Separation of four sulphonic acids as cetrimide ion pairs (cf. Table I for code) on the zirconia column of Fig. 5. Operating conditions as in Fig. 2.

Adsorption LC with this zirconia suggested that its surface polarity (density of strong adsorption sites) is between that of silica and that of the ceria of the earlier study[5]. The position of the retention maxima with respect to cetrimide concentration on Fig. 4 supports this idea, since this maximum lies between $3 \times 10^{-3}$ and $7 \times 10^{-3}$ mol dm$^{-3}$ for ceria (ref. 5, Fig. 3) and for SAS-silica (ref. 7, Fig. 1): the maximum for zirconia packings lies slightly higher than these at $1.4 \times 10^{-2}$ $M$: highest of all are the $k'$ maxima of Fig. 1 at $2.7 \times 10^{-2}$ $M$ cetrimide on silica. Unfortunately, the data from these three studies are not strictly comparable, since different solvent systems were used; however, a short series of experiments confirmed that maximum retention on zirconia with the same solvent used in the earlier work on ceria separations was also at $1.4 \times 10^{-2}$ $M$ cetrimide concentration.
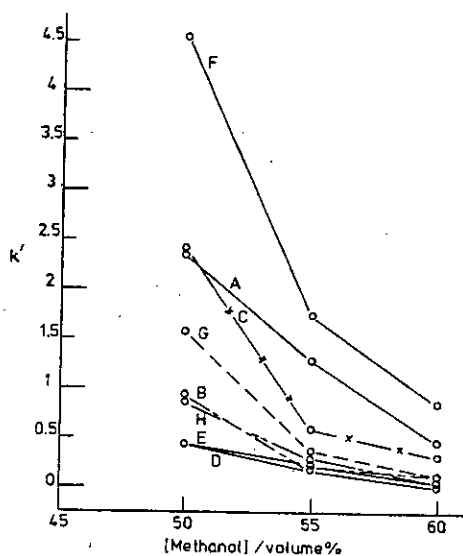


Fig. 7. Relationship of sample retention ($k'$) to volume % methanol in eluent at constant cetrimide concentration ($7 \times 10^{-3}$ mol dm$^{-3}$). Column packing: hydrothermally modified zirconia. Analyte coding as in Table I.

If the mechanism of separation on the oxide gel–cetrimide packings is similar to that of alkyl-bonded silicas as suggested earlier, then the retention of analyte should vary with solvent composition[10,11]. That this is so on zirconia is evident from Fig. 7, in which variation of $k'$ with percentage (by volume) of methanol in the methanol–water eluent (containing $0.7 \times 10^{-2}$ $M$ cetrimide) is plotted for a range of solutes. Note that selectivity also varies with solvent composition, although this latter effect is rather less pronounced than solvent-induced changes in retention.

CONCLUSIONS

Data from this study and that from previous investigations[5,6] on the interaction between cationic surfactant dissolved in the eluent phase and LC column packings prepared from porous gels of cerium(IV) oxide, silicon(IV) oxide and zirconium-

(IV) oxide may be interpreted as arising from the *in situ* generation of a strongly (probably electrostically) bonded alkyl hydrocarbon surface phase. Horvath *et al.* (ref. 7, Fig. 12) have shown that the relationship between concentration of surfactant and $k'$ for a given analyte is a function of both the alkyl chain length of the surfactant (*i.e.*, hydrophobicity) and the polarity (*i.e.*, water content) of the eluent. So, as hydrophobic character of the stationary phase is increased with a constant composition solvent, a corresponding increase in the surfactant concentration at maximum solute retention would be predicted. Such an increase is observed with the silica, zirconia and ceria stationary phases used in this and the preceding study[5]. When this observation is combined with the fact that the relative retention of hydrocarbon solutes in the cetrimide–silica system is very similar to that observed on octadecylsilica, it seems clear that an eluent-generated hydrophobic surface is the main retentive agent in the surfactant–oxide gel systems.

The results reported here suggest that many "reversed-phase" LC separations at present carried out on alkyl-bonded silica gel packings could be done with equal facility on unmodified porous oxide gel packing materials by addition of a suitable surfactant to the typical aqueous–organic eluents of the technique. Both absolute and relative retention of polar and non-polar analytes are shown to be controlled by solvent composition both with respect to the bulk organic components (*i.e.*, methanol, propanol, etc.) and the trace surfactant. Additional control of the parameters affecting a given separation may be obtained by use of oxide gels with differing surface characteristics, as in classical adsorption chromatographic practice. This extension to a wider range of analytes (and a novel zirconia column packing) of the "soap" chromatographic systems described in a study by Knox and Laird[6] of separations of polar sulphonic acids as ion pairs on an alkyl-bonded silica and on silica column packings would appear to be a useful additional to the LC separation modes in current use.

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# HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

## III. NON-IONIC SURFACTANT EFFECTS

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SUMMARY

The effects of addition to aqueous–organic eluents of four members of a group of chemically similar non-ionic surfactants (Tweens) on the chromatographic properties of silicon(IV) oxide gels has been investigated. It was found that small amounts of Tween added to eluent altered the silica surface so that hydrocarbon, phenolic and ketonic eluites were retained to give separations very similar to those obtained with an alkyl ($C_{18}$) covalently bonded silica.

INTRODUCTION

Knox and co-workers[1–3] demonstrated that retention of charged analytes on columns of alkyl-bonded silica gels could be increased by inclusion in the mobile phase of suitably hydrophobic "counter-ions". This technique is often referred to as "soap" or "reversed-phase ion-pair" chromatography. These adducts to the mobile phase in this technique are often charged surfactants such as alkyl sulphonates and sulphates or tetraalkylammonium salts. However, little information is available on the possible effects of addition of non-ionic detergents to these separation systems.

Gilbert and Wall[4] found that porous ceria column packings gave eluite retention from aqueous methanolic eluents containing hexadecyl trimethylammonium bromide similar to that reported by Knox and Laird[1] on SAS-silica, an alkyl-bonded high-performance liquid chromatography (HPLC) column packing. Ghaemi and Wall[5] reported separations of some aromatic hydrocarbons, ketones and sulphonic acids on columns of silica and zirconia modified by dynamic interaction with quaternary ammonium cationic surfactants dissolved in aqueous–organic eluents, and also separations[6] of peptides on columns of silica modified by reaction with solutions of mixtures of non-ionic and anionic detergents.

In the present study the effects of addition of some non-ionic surfactants to aqueous methanolic eluents of silica columns are described. This class of surface-active compound is also shown to be deposited onto the silica gel surface. Furthermore, data presented here show that as the hydrophobicity of the surfactant increases

(*i.e.*, as the hydrophobic carbon chain length increases), retention of aromatic hydrocarbons and ketones also increases.

## EXPERIMENTAL

### Instrumentation

Chromatographic systems were assembled as required from M6000A (Waters Assoc., Milford, MA, U.S.A.) or 110A (Beckman, Irvine, CA, U.S.A.) pumps; CE212 (Cecil Instruments, Cambridge, Great Britain) or SF650 (Applied Chromatography Systems, Luton, Great Britain) ultraviolet absorption detectors; septum injectors and columns (125 × 4.6 mm I.D.) made in the laboratory; and guided plunger microsyringes (Scientific Glass Engineering, Melbourne, Australia).

### Column packings and reagents

The silica gels used in these experiments were Hypersil (Shandon Southern Products, Runcorn, Great Britain; $d_p = 5 \mu$m, $S_{BET} \approx 200$ m$^2$ g$^{-1}$) and GA 43, an experimental spherical material ($d_p = 9 \mu$m, $S_{BET} \approx 170$ m$^2$ g$^{-1}$).

Columns were packed by the "upward slurry" technique described by Bristow *et al.*[7] at 300 bar. The silica packing materials were suspended and packed in methanol. Solvent methanol was either AnalaR grade (BDH, Poole, Great Britain) or HPLC grade (Rathburn Chemicals, Walkerburn, Great Britain). Tweens 20, 40, 60, and 80 were purchased from Sigma (London, Great Britain) and were all described as of industrial quality. No manufacturers' batch numbers were quoted by the vendors.

### Measurement of "adsorbed" surfactant

Samples (*ca.* 1 g) of silica gel (GA 43) were weighed into tared, stoppered flasks and dried *in vacuo* for 30 min at 120°C. Flask and contents were cooled in a desiccator and reweighed to determine the dry weight of the silica. Methanol–water (1:1, 200 cm$^3$) containing a known concentration of surfactant was mixed with the dried silica for 5 min in an ultrasonic cleaning bath to ensure complete dispersion of the particles. The mixture was filtered by suction through a sintered glass funnel and the excess solvent carefully sucked from the retained silica. The "filter-dry" silica was weighed and then dried *in vacuo* at 120°C, cooled in a desiccator and weighed again to obtain both the dry weight and the "wet" weight. In no case was the amount of detergent adhering to "filter-dry" silica as excess *solution* greater than a few per cent of the amount bound via surface interaction.

The above procedure was repeated for each determination with a fresh 200 cm$^3$ sample of surfactant solution to ensure complete equilibration.

## RESULTS AND DISCUSSION

### Adsorption of Tweens

Table I shows that the amount of non-ionic surfactant sorbed on the silica surface is a function of detergent concentration in the contacting solution, as has been shown by Rupprecht[8]. A general tendency to *decreasing* adsorption with increasing hydrophobicity was also observed, which will be discussed later in greater detail. The highest degree of sorption shown in Table I (197 mg of Tween 20 per gram of

TABLE I

SORPTION OF TWEEN ON GA 43 SILICA

| | $10^3[Tween]/kg\ dm^{-3}$ | Tween adsorbed (mg per g $SiO_2$) | Hydrophobic alkyl chain |
|---|---|---|---|
| Tween 20 | 2.0 | 123 | $(CH_2)_{11}CH_3$ |
| | 3.0 | 197 | |
| Tween 40 | 2.1 | 65 | $(CH_2)_{15}CH_3$ |
| | 4.3 | 150 | |
| Tween 60 | 2.2 | 52 | $(CH_2)_{17}CH_3$ |
| Tween 80 | 5.0 | 85 | $(CH_2)_8CH = CH(CH_2)_7CH_3$ |

$SiO_2$) corresponds to a surface coverage of *ca.* 1.5 $\mu$mol m$^{-2}$, which is about half the value reported by Roumelotis and Unger[9] for $C_8$ and $C_{18}$ alkyl-bonded silicas.

*Separations on silica gel*

Methanol–water (1:1) was used as the eluent base in all experiments reported here, with addition of the appropriate Tween to give concentrations between 0.5 and
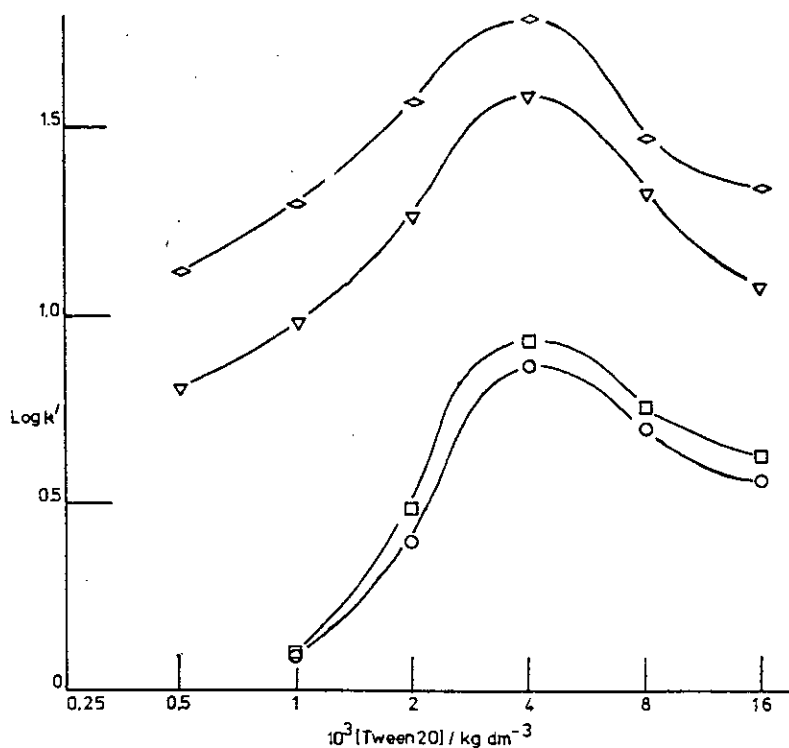


Fig. 1. Relationship of log $k'$ to concentration of Tween 20 in methanol–water (1:1) eluent. Column packed with Hypersil ($d_p \approx 5 \mu$m). Flow-rate 1 cm$^3$ min$^{-1}$ at ambient temperature. Eluites: O = 9-fluorenone; □ = naphthalene; ▽ = anthracene; ◊ = pyrene.

$16 \times 10^{-3}$ kg dm$^{-3}$. All separations were carried out at ambient temperature (15–23°C) with an eluent flow-rate of 1 cm$^3$ min$^{-1}$.

Passage of between 100 and 150 cm$^3$ of solvent containing Tween 20 was necessary to completely equilibrate a column (125 × 4.6 mm I.D.) packed with Hypersil. Fig. 1 shows the dependence of the capacity factor, $k'$, of some aromatic hydrocarbons and ketones on the Tween 20 modified silica gel columns as a function of detergent concentration. Fig. 2 shows the same retention/[surfactant] relationship for Tween 80, which has an unsaturated carbon chain.

The composite graph, Fig. 3, shows that the Tween concentration at which maximum retention is observed for two eluites is a function of surfactant hydrophobicity. As would be expected by analogy with alkyl-bonded silicas[10], the largest values of $k'$ are observed with the most hydrophobic Tween, and, moreover, the surfactant concentration needed for maximum retention is progressively *reduced* as the carbon chain length is increased. Hemetsberger et al.[10] showed that $k'$ was a linear
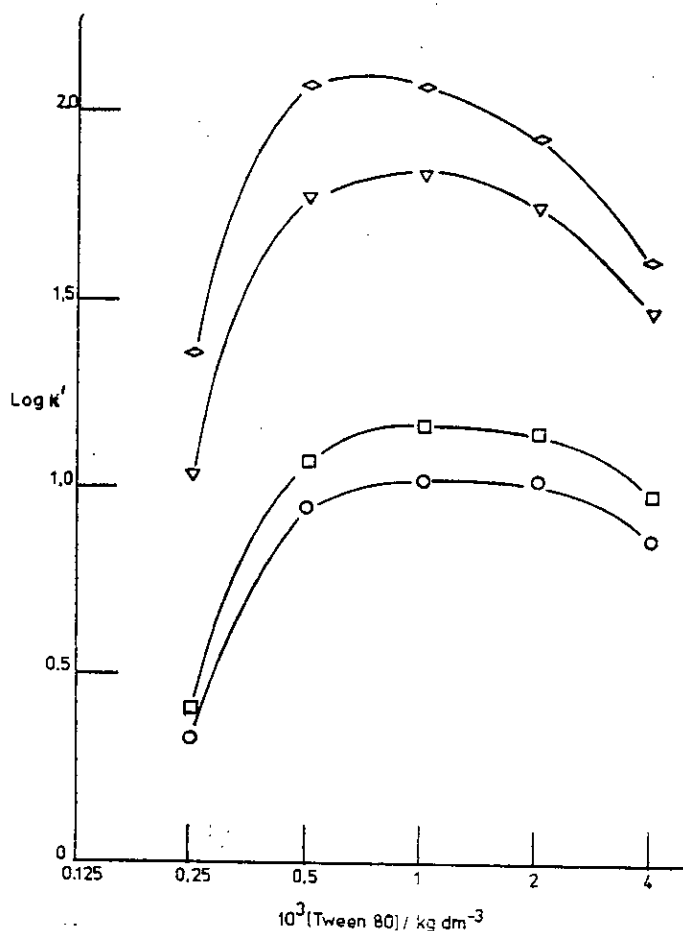


Fig. 2. Relationship of log $k'$ to concentration of Tween 80 in eluent. Chromatographic conditions and eluites as in Fig. 1.
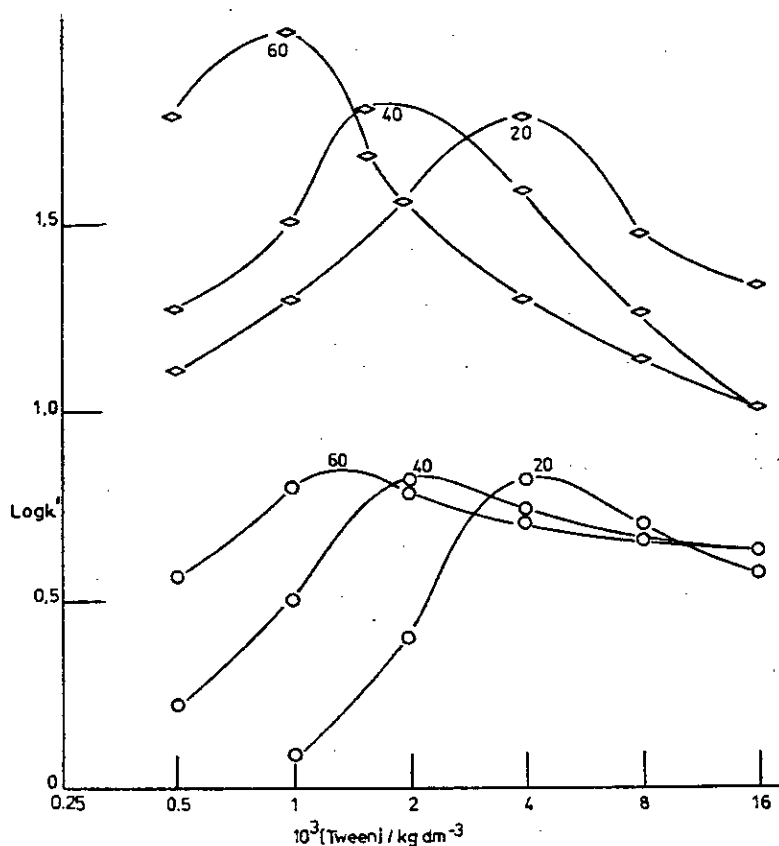
Fig. 3. Relationship of log $k'$ to concentration of Tweens 20, 40, and 60 in the eluent. Samples and conditions as in Fig. 1.

function of percentage carbon content on a series of alkyl methyl silyl "bonded-phase" packings, and a similar trend is observed with these dynamically coated silica surfaces.

In view of the observations by Hemetsberger[10] on the relationship of eluite retention to chain length of *bonded* alkyl residue, one would expect $k'$ values measured in those surfactant solutions giving maximum retention induced by that surfactant to increase by a relatively large factor for an increase of two or four methylene groups in the alkyl chain length of the detergent molecule. However, it can be seen from Table I that surface sorption of Tweens varies *inversely* as chain length. So the greater hydrophobicity of the longer chains is counterbalanced by lower surface coverage of the hydrophilic silica. Fig. 3 demonstrates that either no increase in $k'$ (fluorenone) or a relatively minor increase in $k'$ (pyrene) is observed with increasing hydrophobicity of the surfactant. It is noteworthy that introduction of a single double bond into the 18-carbon chain of Tween 60 to produce Tween 80 (compare Figs. 2 and 3) significantly increased retention of all eluites shown, but did not apparently change the detergent concentration at which maximum $k'$ was observed.

Figs. 4 (Fig. 4a is without surfactant and Fig. 4b with surfactant) and 5 are representative chromatograms of aromatic hydrocarbons, phenols and ketones on silica. Separations of one of the standard test mixtures suggested by Knox[11] for determination of the efficiency of columns of alkyl-bonded silica columns are shown in Figs. 6 and 7. The order of elution and relative retention of the analytes on the ODS-Hypersil column (Fig. 6) is very similar to that observed in aqueous methanolic Tween 40 elution from a silica column (Fig. 7).

The similarity in elution order and relative retention of the various analytes shown in Figs. 6 and 7 suggests a similarity in separation mechanism. As suggested in earlier studies in this series, the result of interaction between silica and the polyol terminus of the Tween molecule is probably a modified surface bearing "brushes" of surfactant molecules. As the concentration of surfactant in the eluent is increased, two competing processes occur. Firstly, coverage of the silica by "brushes" increases and, secondly, association of "bound" and free surfactant molecules (probably by hydrophobic bonding of alkyl chains) also increases.
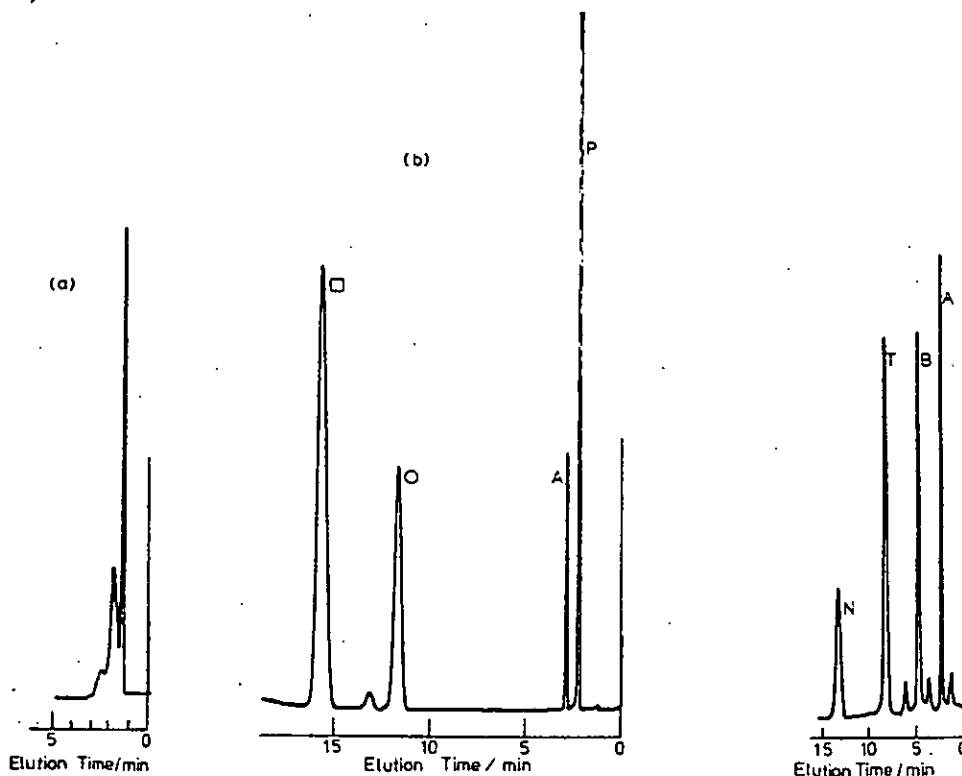


Fig. 4. (a) Demonstration of minimal retention of sample mixture of Fig. 1 on Hypersil from methanol–water (1:1) solvent system in absence of surfactant. (b) Separation of fluorenone (O) and naphthalene (□) on column of Fig. 4a in equilibrium with a solution of Tween 40 ($2 \cdot 10^{-3}$ kg dm$^{-3}$) in methanol–water (1:1). Less retained eluites are acetophenone (A) and phenol (P), respectively.

Fig. 5. Separation of acetophenone (A), benzene (B), toluene (T), and nitrobenzene (N). Column, eluent, and conditions as in Fig. 4b.
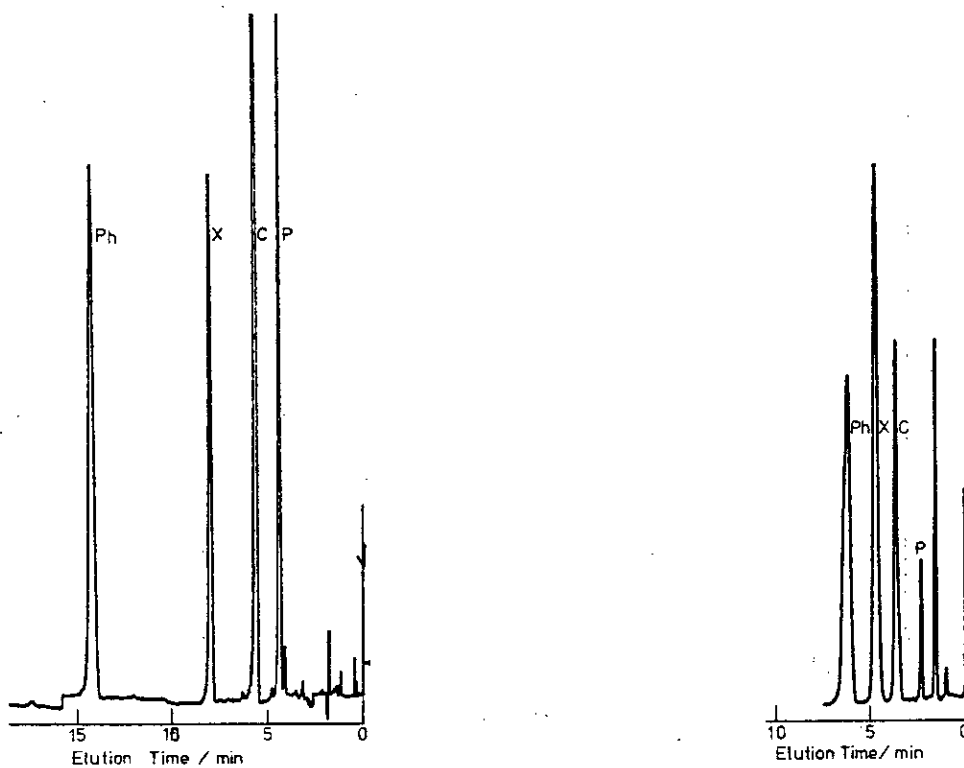
Fig. 6. Separation of phenol (P), *para*-cresol (C), 3,5-xylenol (X), and phenetole (Ph) on a column (125 mm × 4.6 mm I.D.) of ODS-Hypersil ($d_p \approx 5 \mu$m). Methanol–water (6:4) was passed through the column at ambient temperature at 1 cm$^3$ min$^{-1}$.

Fig. 7. Separation of sample mixture as shown in Fig. 7 with column and solvent system as in Fig. 4b.

The consequence of the first process would be an increase in retention of hydrophobic solutes with increasing brush density, but the second process would lead to an increasingly *polar* surface, studded with brushes with polyol termini instead of aliphatic hydrocarbon chains. Horváth et al.[12] have shown that the elution order of solutes from columns of alkyl-bonded silica packing materials is largely a function of the balance of surface and solute hydrophobicities. Accordingly, since essentially the same elution order is observed from the Tween–silica system as from octadecyl silica packings, retention in the former system should be largely due to the same properties of solute and surface.

If this hypothesis does accurately reflect the retention mechanism, then a steady increase in retentive power for chemically similar analytes should be observed with increasing brush density. This increase in retentive character will be counter-balanced by a decline in brush hydrophobicity which would ultimately lead to a decline in $k'$ as surfactant concentration is increased. This behaviour has been observed with all single surfactant systems examined so far (*cf.* Figs. 1, 2 and 3 and refs. 1, 4 and 5), but may not be the case when ionic and non-ionic surfactants are used in combination[6].
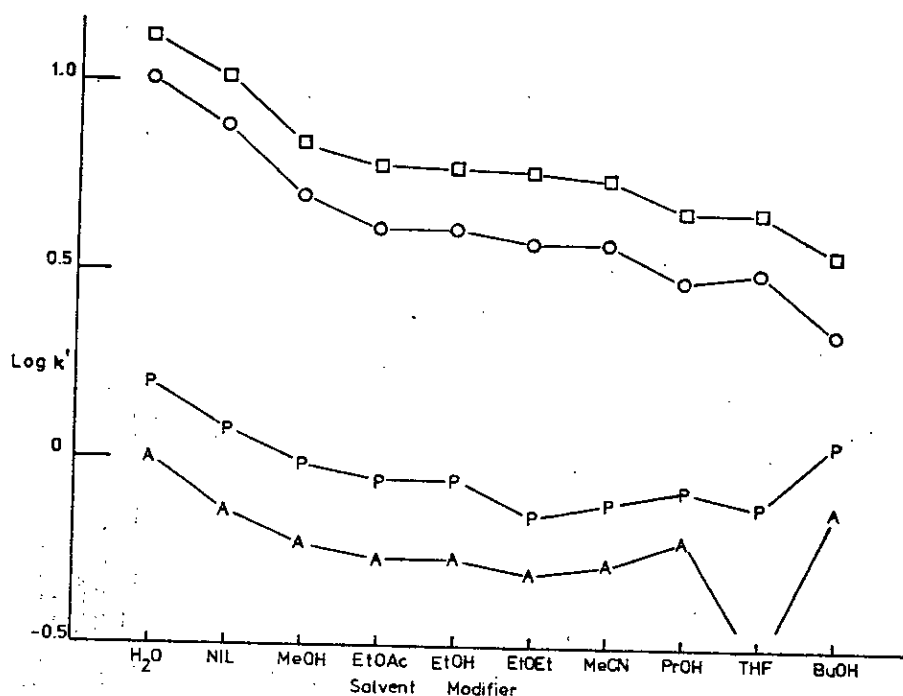
Fig. 8. Relationship of log $k'$ to 10% (v/v) addition of a range of solvent modifiers. Conditions as in Fig. 4b, except solvent now 10% additive plus 90% [methanol–water (1:1) containing Tween 40 $(2 \cdot 10^{-3} \text{ kg dm}^{-3})$]. NIL = No addition; MeOH = methanol; EtOAc = ethyl acetate; EtOH = ethanol; EtOEt = diethyl ether; MeCN = acetonitrile; PrOH = propan-1-ol; THF = tetrahydrofuran; BuOH = butan-1-ol.

Control of both absolute and relative retention of several different eluites is usually maintained by choice of organic modifier and its concentration in "reversed-phase" chromatography. Tanaka et al.[13] and Bakalyar et al.[14] have clearly shown that selectivity in this LC mode is critically dependent on the nature and proportion of organic modifier in the eluent system. Fig. 3 is a composite diagram showing the effects on retention of four eluites of small changes in eluent composition at constant surfactant concentration. It shows that, as an added third solvent component decreases in polarity, there is a general trend to more ready elution. However, as would be expected by analogy with alkyl-bonded silica separations, the effects of solvent change are by no means equal on all four solutes. Clearly, control of organic modifier nature and proportion allows considerable control of selectivity in any separation attempted in this new HPLC mode.

CONCLUSIONS

Previous investigation[1,4,5] of the interaction between cationic surfactant and/or mixed non-ionic–anionic surfactants[6] dissolved in the eluent phase and LC column packings prepared from porous silicon(IV) and zirconium(IV) oxides and the present data on similar interactions of non-ionic surfactants with silicon(IV) oxide have

shown that this new mode of HPLC may be used to separate a wide range of analytes varying from sulphonic acids through neutral aromatic hydrocarbons and ketones to basic amines, amino acid esters, and peptides.

Selectivity in these separations may be enhanced by control of pH, solvent composition, ionic strength, chemical nature of the oxide surface, and selection of the appropriate surfactant. In the present work, Tweens (alkyl sorbitan polyethylenoxy polyols) were shown to be strongly bonded to silica gel surfaces; indeed, they proved to be impossible to wash off the column packing with several litres of water or aqueous alcoholic solvents. This technique of separation is rapidly and easily set up, and many of the GPLC separations presently done by "reversed-phase" chromatography on alkyl-bonded silicas could be carried out with equal efficiency using this new oxide–surfactant approach.

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# HYDROPHOBIC CHROMATOGRAPHY WITH DYNAMICALLY COATED STATIONARY PHASES

## IV. Anionic Surfactant Effects on Alumina

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## SUMMARY

The effects of addition to aqueous methanolic eluents of two alkyl sulphate surfactants on the chromatographic properties of aluminium III oxide gels have been studied. It was found that the basic active sites on the alumina surface interacted with anionic surfactants in a fashion analogous to that previously observed with acidic active centres on silica and cationic surfactants.

# INTRODUCTION

Knox and Laird (1) reported that aromatic sulphonic acids
could be retained and separated on columns packed with porous
silica gel particles when a cationic surfactant was added (in
low concentration) to the aqueous/organic eluting solvent system.
These findings were amplified and extended to non-polar eluites
separated on columns packed with cerium IV oxide, silicon IV
oxide, and zirconium IV oxide in the studies of Gilbert and
Wall (2) and Ghaemi and Wall (3).

Since dynamic coating of cationic surfactants clearly gave
analytically useful hydrophobic column packing materials from
hydrophilic porous acidic oxide gels, clarification of the
nature of the interaction which produced the hydrophobic surface
became necessary.    Extension of the surfactant range to non-
ionic and anionic types appeared to be an obvious experimental
route.    A report on the first of these two types by Ghaemi
and Wall (4) apparently confirmed the initial hypothesis (3)
that strong interactions between the polar oxide surface and
the polar "end" of the surfactant provided the means of binding
a partial monolayer of hydrophobic alkyl chains to that surface.
However, as mentioned in a further study of 'Dynamic Soap
Chromatography' of peptides by Wall (5), anionic detergents do
not so react with acidic oxide surfaces in the polar aqueous
eluents used.

Accordingly, this present study describes the effects of
addition of anionic (and nonionic) surfactants to aqueous methanolic
eluents of columns packed with aluminium III oxide gel particles.
These commercially available γ-alumina packings are known to

possess basic as well as acidic adsorption sites, and may be shown to adsorb and retain small amounts of anionic surfactants from external aqueous methanolic solutions. Furthermore, as has been previously demonstrated with cationic and nonionic detergents, retention of both charged and uncharged eluites increases with increasing alkyl carbon chain length of anionic detergent. Note, however, that in aqueous "micellar" solutions of high surfactant concentration, nonpolar analytes were shown by Armstrong and Terrill (6) to be less strongly retained on the alumina/surfactant interface.

## EXPERIMENTAL

### Instrumentation

Chromatographic systems were assembled from components as required as outlined in an earlier report (4).

### Column Packings and Reagents

The porous alumina used in the majority of these experiments was Spherisorb (Phase Separations, Ltd., Queensferry, U.K., AlOX grade, $dp \simeq 10\mu m$, $S_{BET} \simeq 93 \ m^2g^{-1}$ and A2OY grade, $dp \simeq 20\mu m$, $S_{BET} \simeq 93m^2g^{-1}$), although Lichrosorb AloxT (E.Merck A.G., Darmstadt, Germany, $dp \simeq 10\mu m$, $S_{BET} \simeq 70m^2g^{-1}$) was used in some confirmatory studies. Until the alumina particles were subjected to calcination in air (24 hours at $600^{O}C$), it proved difficult to get fully reproducible retention results from column to column.

Columns were packed by the "upward slurry" techniques described by Bristow, et al (7) at 300-500 bar constant pressure, using methanol both for packing and suspension of the alumina particles. Solvent methanol was either AnalaR grade (BDH

Chemicals Ltd., Poole, England) or HPLC grade (Rathburn

Chemicals Ltd., Walkerburn, Scotland) as required.    Sodium

laurylsulfate (SDS), "puriss," and Tergitol 7 (T.7), sodium

"heptadecyl" sulfate were purchased from Fluka A.G., Buchs,

Switzerland.    Tween 40, stated to be "industrial quality" was

obtained from Sigma Chemicals, Ltd., London, England.    All

other solvents and reagents used were of Reagent grade and

were used as received from the suppliers.    Water was distilled

from glass.


RESULTS  AND  DISCUSSION


Figure 1 here

Figure 1 shows that the amount of Tergitol 7 sorbed on the

alumina surface is a (non-linear) function of detergent concentration

in the contacting solution, as has been shown by Rupprecht (8) and

Scott and Kucera (9).    Surface coverage was measured both by a

column breakthrough  technique (cf Knox and Laird, 1) and by a

procedure of constant withdrawal and addition (of an aliquot of

concentrated surfactant) to a suspension of alumina in the 1:1

aqueous methanol eluent base.    However, since the Tergitol 7

active principle is anionic, a cationic dye must be used for the

colorimetric (1) estimation of detergent concentration.    Methylene

blue was so used in these experiments, although the relationship

of surfactant-dyestuff ion-pair concentration to absorbance @ 656nm

was distinctly non-linear.    The highest degree of sorption

measured (at $3.2 \times 10^{-2}$M [T, 7]) showed that there were ca 1.1µmoles

of detergent on each square metre of the alumina surface.    The

best coverage of silica by covalently bonded mono-layer alkyl

chains of equivalent length was shown by Unger, et al (10) to be

roughly three times as dense as that above.

## Surfactant-Retention Relationships

A 1:1 volume/volume solution of methanol and water was used as the eluent base in all the present experiments. Solid surfactant (SDS) or a concentrated solution in the 1:1 aqueous methanol of liquid surfactant (T.7 and Tween 40) was added as appropriate to this base. All separations were done at ambient temperature (15-23°C) with an eluent flow rate of 1 cm$^3$min$^{-1}$. Quite large volumes (200-500 cm$^3$) of the least concentrated solutions of surfactants used in this study had to be passed through the packed columns before constant retention values could be observed. In all these surfactant/oxide gel systems there is apparently a very slow equilibration process occurring after the initially rapid uptake of sorbed detergent. Accordingly, if a column is to be prepared for a <u>practical</u> analytical separation, it is possible to save working time by establishment of partial equilibrium with a concentrated solution before equilibrating with the final, usually more dilute eluent.

Figs 2 and 3 here

Figure 2 demonstrates the dependence of the capacity factor, $k' = \dfrac{t_{retention} - t_{unretained}}{t_{unretained}}$, of some non-polar aromatic compounds on the SDS modified alumina column as a function of surfactant concentration. The form of this relationship, rising through a maximum (at [SDS] $\approx 3\times10^{-2}$mol.dm$^{-3}$) and declining thereafter with increasing solvating power (surfactant concentration), closely resembles the earlier data with the same elutes on cationic (3) and nonionic (4) surfactant-modified silicas. Furthermore, both the order of elution and the relative retention of these analytes are also similar to those observed in these earlier studies and on columns of alkyl bonded silica, suggesting strongly that there is a common mechanism of retention; i.e. hydrophobic

<u>NEW LEGEND</u>

Figure 4   Variation in retention as a function of nonionic surfactant
           (Tween 40) concentration.    Analytes as in Figure 2 except
           Δ-<u>meso</u>benzanthrone


<u>CHANGES TO P 5</u>

delete:   Sentence beginning line 17 "Interestingly.....................
          chromatography". (on line 21)

insert:   Alumina adsorbents are usually considered to have a more
          reactive, polar surface than those based on silica gel
          and perhaps residual surface polarity reduces the tendency
          of hydrophobic analytes to be retained at the Tween 40/
          Alumina interface.    Certainly this range of eluites (and
          several others not shown) yields $K^{\ell}$ values about half of
          those measured in earlier studies (4) on silica/Tween 40
          column systems.    However, the surfactant concentration
          at which maximal retention is observed is essentially the
          same as that found earlier in the silica column study.

interaction.

This conclusion is reinforced by the data presented in Figure 3, in which the same K'vs [surfactant] relationship is displayed for a Tergitol 7-alumina system. Since T.7 is an alkyl sulphate differing only in chain length from SDS ("$C_{17}$" as opposed to $C_{12}$), the evident increase in maximum retention, achieved at lower T.7 concentration accords well with the work of Hemetsberger, et al. (11) on the effects of alteration of (covalently bonded) alkyl chain length in hydrophobic chromatography, and the extended discussion by Horvath et al. (12) of (alkyl sulphate) ion-pair chromatography on alkyl bonded silicas.

Figure 4 here

The same picture of retention rising to a maximum and then falling with increasing surfactant concentration is also shown in Figure 4, which represents the effects of a nonionic surfactant, Tween 40, on the chromatographic properties of the alumina surface. Interestingly, although K' for pyrene in this system is roughly one tenth of that given by a silica - Tween 40 system (4), the concentration of the surfactant in the contacting eluent at maximum retention is very similar to that found earlier for silica column chromatography. This latter finding suggests that the dominant feature governing maximum sample retention is the solvating power of the eluent rather than the density of surface coverage by sorbed surfactant molecules. Further work will be necessary to clarify this point.

Mechanism of Retention of Cationic Eluites

The above data support the hypothesis that the mode of retention and separation of nonpolar analytes on alumina-surfactant

column systems is qualitatively similar to that observed with
silica-surfactant and alkyl bonded silica column systems.
However, when cationic samples were examined on alumina-SDS
(and T.7) columns, puzzling results were obtained.   The mixed
nonionic-anionic surfactant separations of peptides reported
earlier (5) were shown to have at least some of the pH and counter-
ion dependence to be expected if a cation exchange process were
operating.    As pointed out in this earlier work, K' for a
particular cationic substance should be inversely proportional
to the concentration of added (counter) cation if retention is
due primarily to ion exchange processes.

<center>Figs. 5 and 6 here</center>

Figure 5 shows that whatever the mechanism for retention of
aralkylammonium ions may be, it is apparently not a simple ionic
exchange between mobile and stationary phases in the column.
The relationship demonstrated between K' and $1/[Na^+]$ has a negative
slope over most of the concentration range tested, but reverses
to a positive slope at higher salt levels.  Apparently these
relatively hydrophobic ammonium ions (or their ion pairs with
alkyl sulphate anions) are retained primarily by hydrophobic
interaction with the hydrocarbon side chains of surfactant
molecules sorbed on the alumina surface.   This conclusion is
borne out by comparison of the data given in Figure 6 with the
similar plot given as Figure 4 of the earlier study (5).  Clearly
the changes in retention in this present study with increasing
salt are similar for both nonpolar and ammonium ion analytes up
to ca $5 \times 10^{-2}$M $[Na^+]$, but the large decrease in retentive power
at even higher salt concentration was not observed in the earlier
studies on the silica-nonionic-anionic surfactant system.

However, these two sets of data are not strictly comparable, since the oxide-surfactant systems and contacting liquids are not the same.

Because the effects of added salt on retention of cationic analytes did not support a cation exchange mechanism, it seemed of interest to examine the effects of changes in acidity of eluants. The variation of pH was achieved by addition of mineral acid, $H_3PO_4$, to a solvent system in which methanol, surfactant, and sodium ion concentrations were held constant, so that all variables except $[H^+]$ and ionic strength were controlled. Similar types of experiment were shown by Hamilton, et al (13,14) to lead to a steady decrease in retention of cationic analytes (amino acids) on polystyrene sulphonic acid exchanges with increasing pH over the range from 2-6. Figure 7 shows just such a relationship for cationic analytes on an alumina-T.7 column system.

Figure 7 here

Since the ammonium ion analytes of this present study are at least 99% in the ionised form over the whole of the observed pH range, no simple explanation seems possible. However, this behaviour is typical of the pattern observed with classical ion exchangers, and retention in the present system is accordingly best described as a balance of ion exchange and hydrophobic bonding. If the density of alkyl chains on the alumina surface was proportional to $[H^+]$, that could account for the results described by Figure 7. No data on the pH sensitivity of anionic surfactant binding to alumina are presently available, so the question must be left open.

## Application

This new technique extends the sampling range amenable to analysis by oxide gel-surfactant chromatography in that cationic solutes will be well retained (under control by variation of pH, surfactant concentration, counter-cation concentration, and organic modifier concentration of the eluent system) in operating conditions such that acidic, zwitterionic, and neutral analytes will also be retained (according to their hydrophobic binding capacities). The more complex eluting solvent systems discussed in the earlier study (5) on "dynamic ion exchange" of tyrosinyl peptides would generate the same analytical capabilities, but as shown above, the retention mechanisms are different in these two procedures, hence their selectivities for a given analysis will also differ.

<center>Figures 8 and 9 here</center>

Figure 8 is a record of a specimen separation of four neutral analytes on an alumina-SDS column. The efficiency of this separation was tested at eluent flowrates of 1.0, 0.8, 0.6, 0.4, and 0.2 $cm^3$ $min^{-1}$ and was shown to increase steadily (the number of theoretical plates increased from 3000 to 4000 over this eluent flow range for the unretained acetone peaks and from 750 to 3000 for the fluorenone peak) with decreasing speed of elution. Estimation of diffusion constants by the Wilke-Chang (15) approximation and calculation of reduced parameters as outlined by Knox (16) suggested that reduction of eluent flowrate to 0.1 $cm^3 min^{-1}$ would have been necessary to achieve optimal column efficiency for separation of 9-fluorenone. However, the above data show that this newest mode of oxide gel-surfactant LC is capable of giving separation efficiencies comparable to those obtained with more conventional techniques.

This assertion ⁱˢ reinforced by examination of Figure 9, which is

a record of the separation of three fully protonated amines with the

same column/eluent system used to produce Figure 8.   In the absence

of surfactant the aqueous methanol eluent base would have eluted all

the above analytes at or near the solvent front, and indeed the

three cationic eluites would be little retained on columns of alkyl-

bonded silica from ⁺ʰⁱˢ a mobile phase at pH3.   The observation of

pronounced retention of quaternary ammonium analytes as well as of

aralkyl ammonium ions suggests that although a purely ion exchange

mechanism cannot be supported (cf. Figure 5) for these ionic eluites,

there must be a substantial element of ion exchange or ion pair parti-

tion in the retentive processes.


CONCLUSIONS

The present investigation confirms the potential and versatility

of a new approach to hydrophobic and ion exchange chromatography

based on dynamic generation of a retaining surface on porous oxide

gel column packing materials.   Moreover, it has shown that the

surface layer(s) generated by direct (electrostatic ?) interaction

of an anionic surfactant with a basic aluminium III oxide differs

significantly from the product of an acidic silicon IV oxide and

mixed nonionic-anionic surfactants.

Chromatographic systems based on those described above

are readily set up, and are clearly versatile, since analyte

retention is controlled (primarily) by eluent composition

variables, i.e., pH, ionic strength, organic modifier type and

concentration, and surfactant type and concentration.   Since

the mechanism of retention of cationic analytes appears to have

both hydrophobic and ion exchange components, variation of

operating temperature should alter selectivity of separation

as well.    Further study is required before a greater under-

standing of the detailed mechanism(s) of analyte retention in this

new "dynamic" mode of liquid chromatography can be attained, but

its value as an additional tool in the analytical workshop is
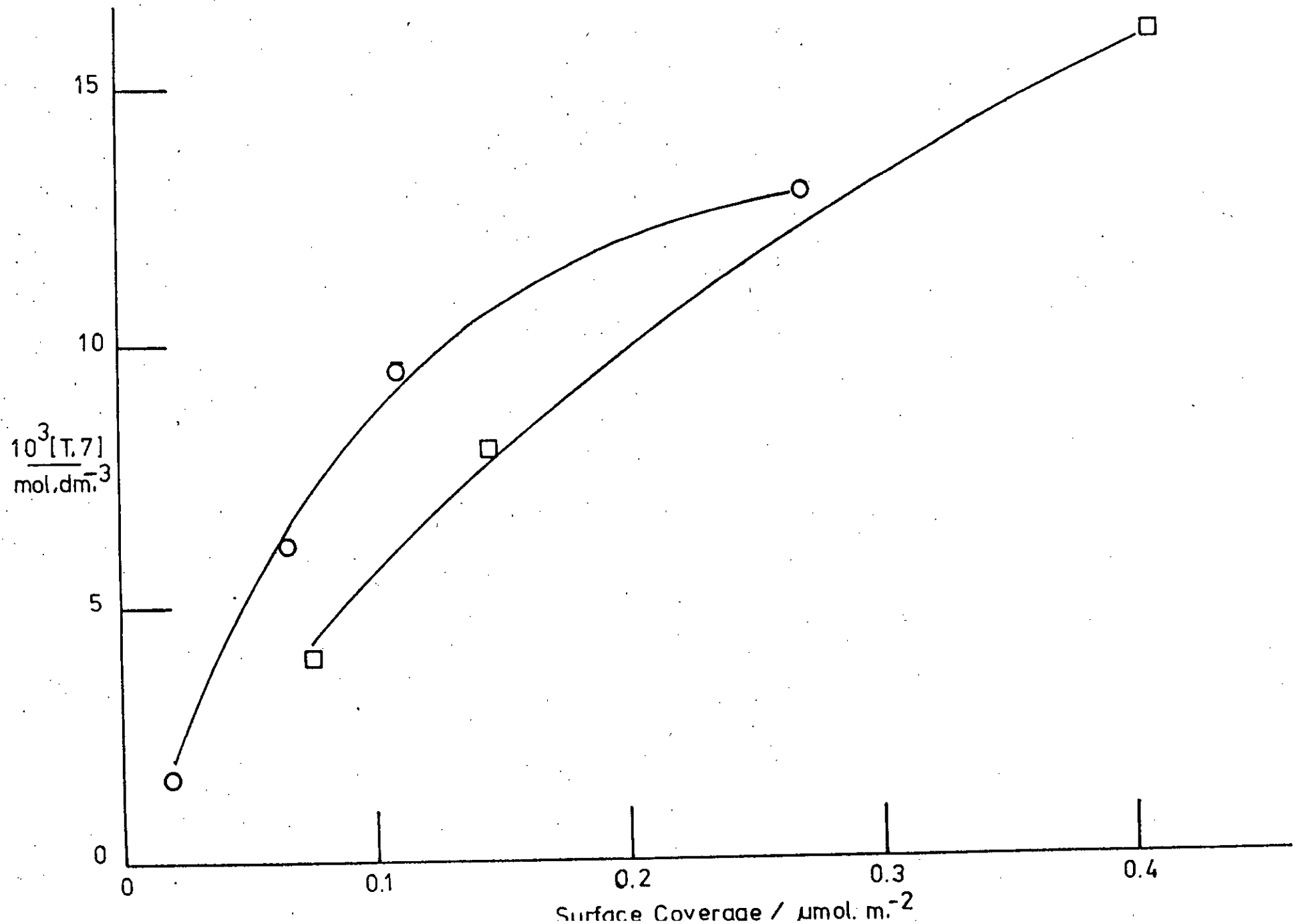
apparent.

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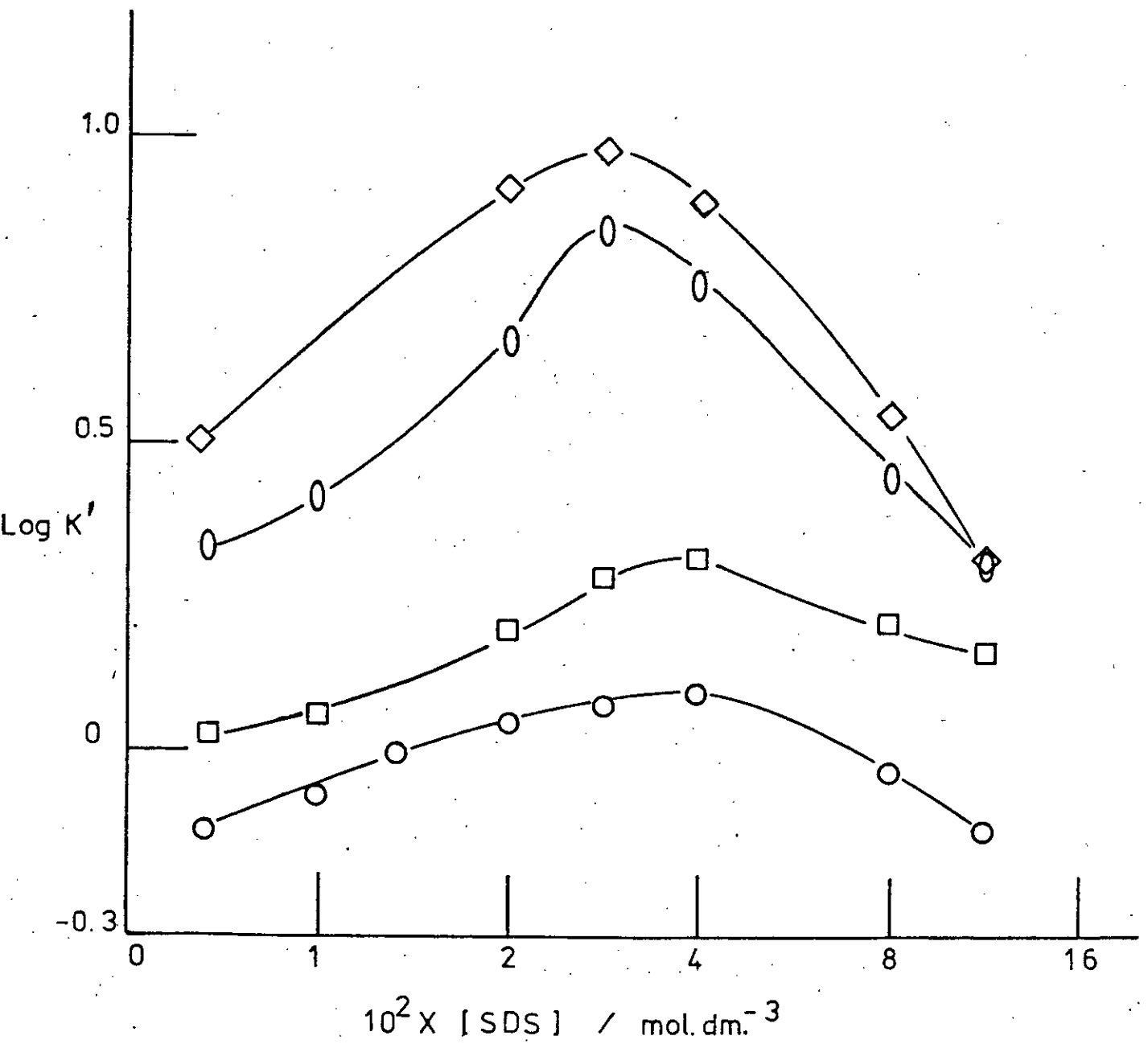LEGENDS FOR FIGURES

1. Surface coverage of alumina by Tergitol 7 ("$C_{17}$" alkyl sulphate). ○-Lichrosorb Alox T; ☐-Spherisorb AlOY.

2. Variation in retention with change in SDS concentration. ○- 9-fluorenone; ☐- naphthalene; ()- anthracene; and ◇- pyrene.

3. Variation in retention with Tergitol 7 concentration. Analytes as in figure 2.

4. Variation in retention of pyrene with Tween 40 (nonionic surfactant) concentration.

5. Effects of change in counter-ion (sodium) concentration on retention at constant ($1.6 \times 10^{-2}$ mol/dm$^3$) concentration of Tergitol 7. ○-: tyrosine methyl ester; ▽- 1-amino-1-phenylethane; ☐- naphthalene; and △- 1-amino-1-(1-naphthyl)ethane.

6. Same data as in figure 5 but plotted as a linear function of counter-ion concentration.

7. Effect of change of pH on retention at constant counter-ion and tergitol 7 concentrations. Analytes as in Figure 5 except ○ - tryptophan methyl ester.
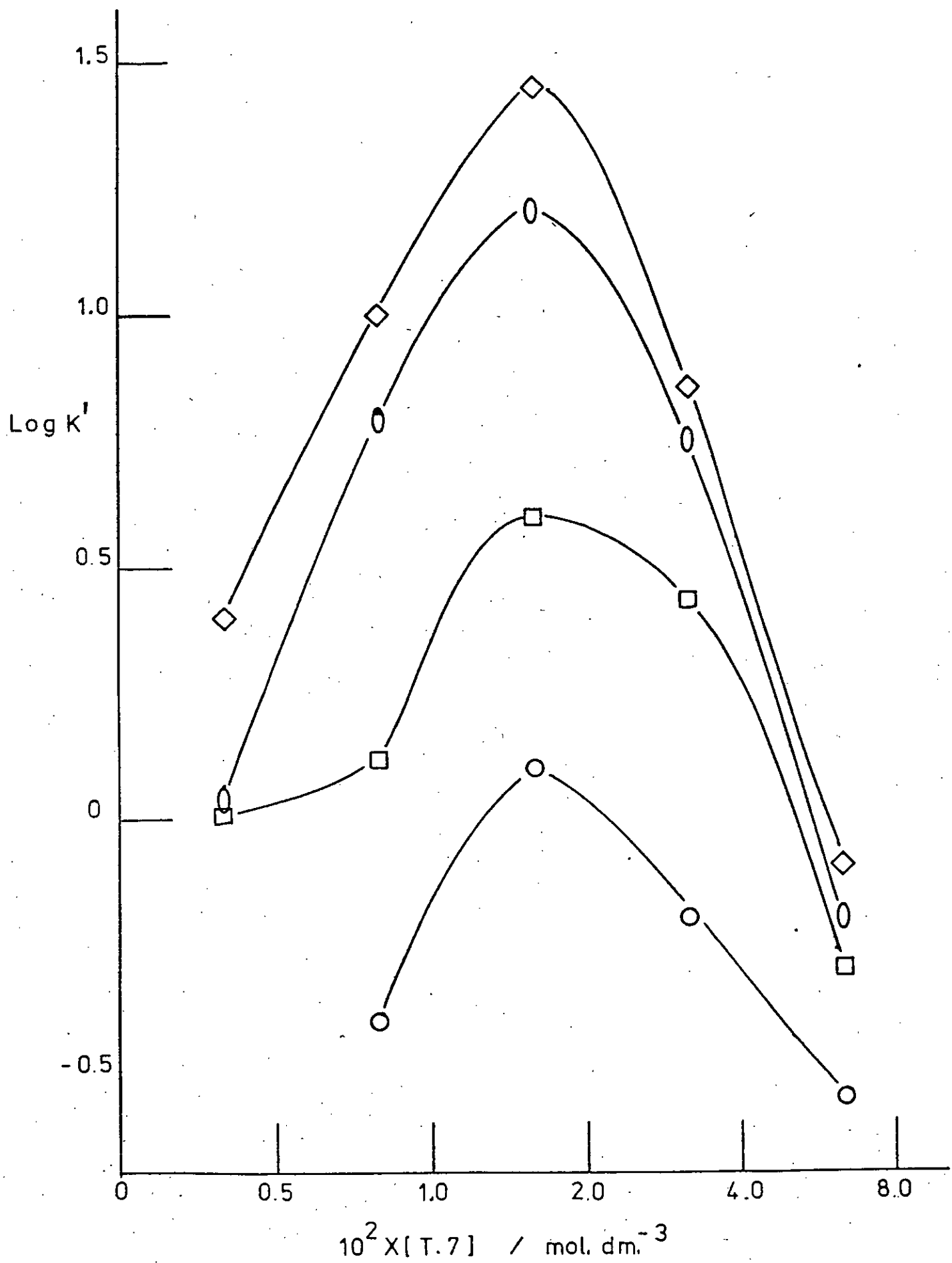
8.  Specimen separation of a mixture of neutral, unionised
    analytes.   Column packed with Spherisorb AlOY, 125x04.6mm;
    flowrate 1 cm$^3$/min of 1:1 methanol/water containing 3.2x10$^{-2}$
    mol/dm$^3$ SDS (pH adjusted to 3.0 by addition of phosphoric
    acid).   Analytes in order of elution are acetone, 2,3-
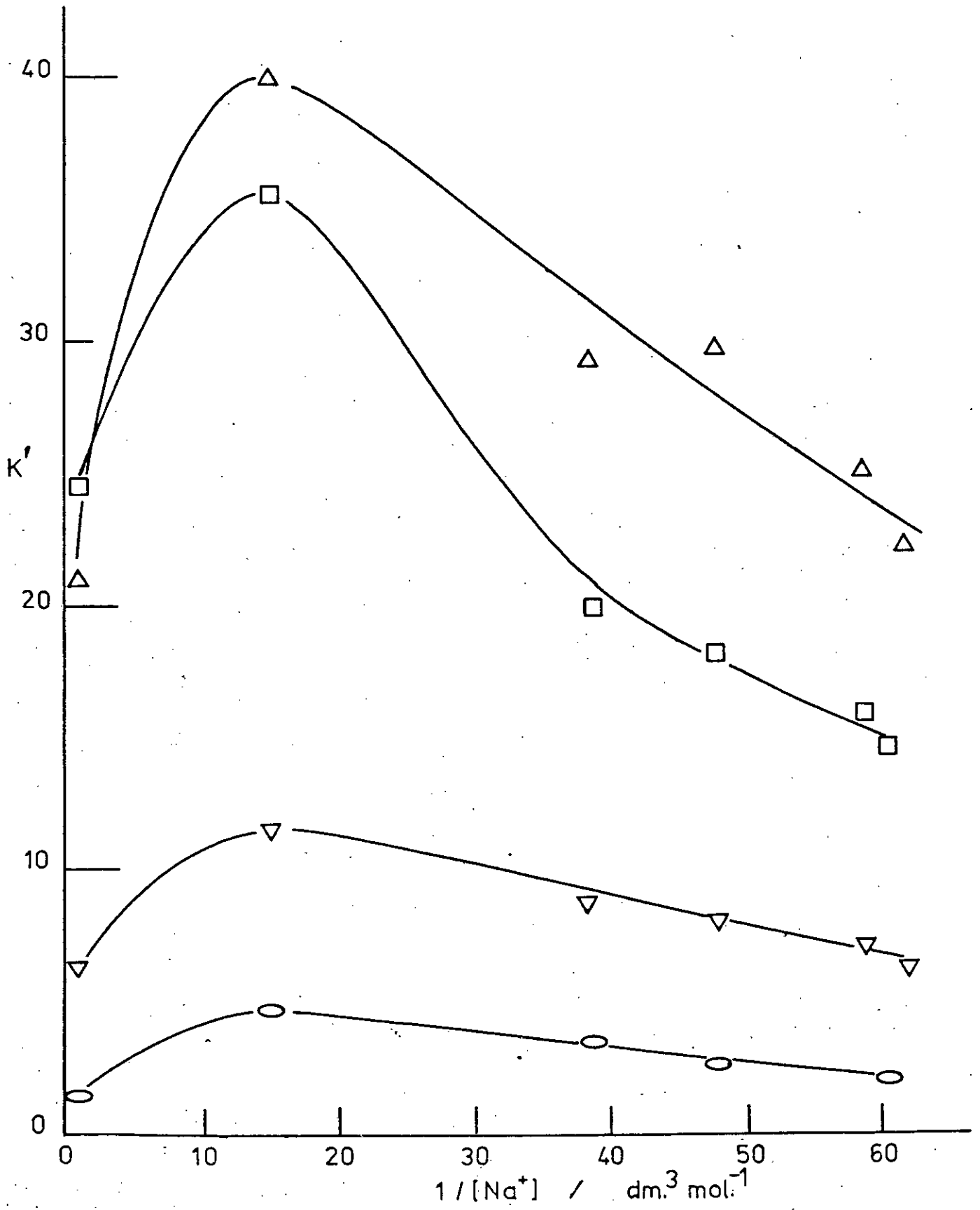    xylenol, 9-fluorenone, and naphthalene.

9.  Specimen separation of cationic analytes on same column as
    in figure 8.   Eluent as in figure 8, but analytes in order
    of elution are;   three impurities, 1-amino-1-phenylethane,
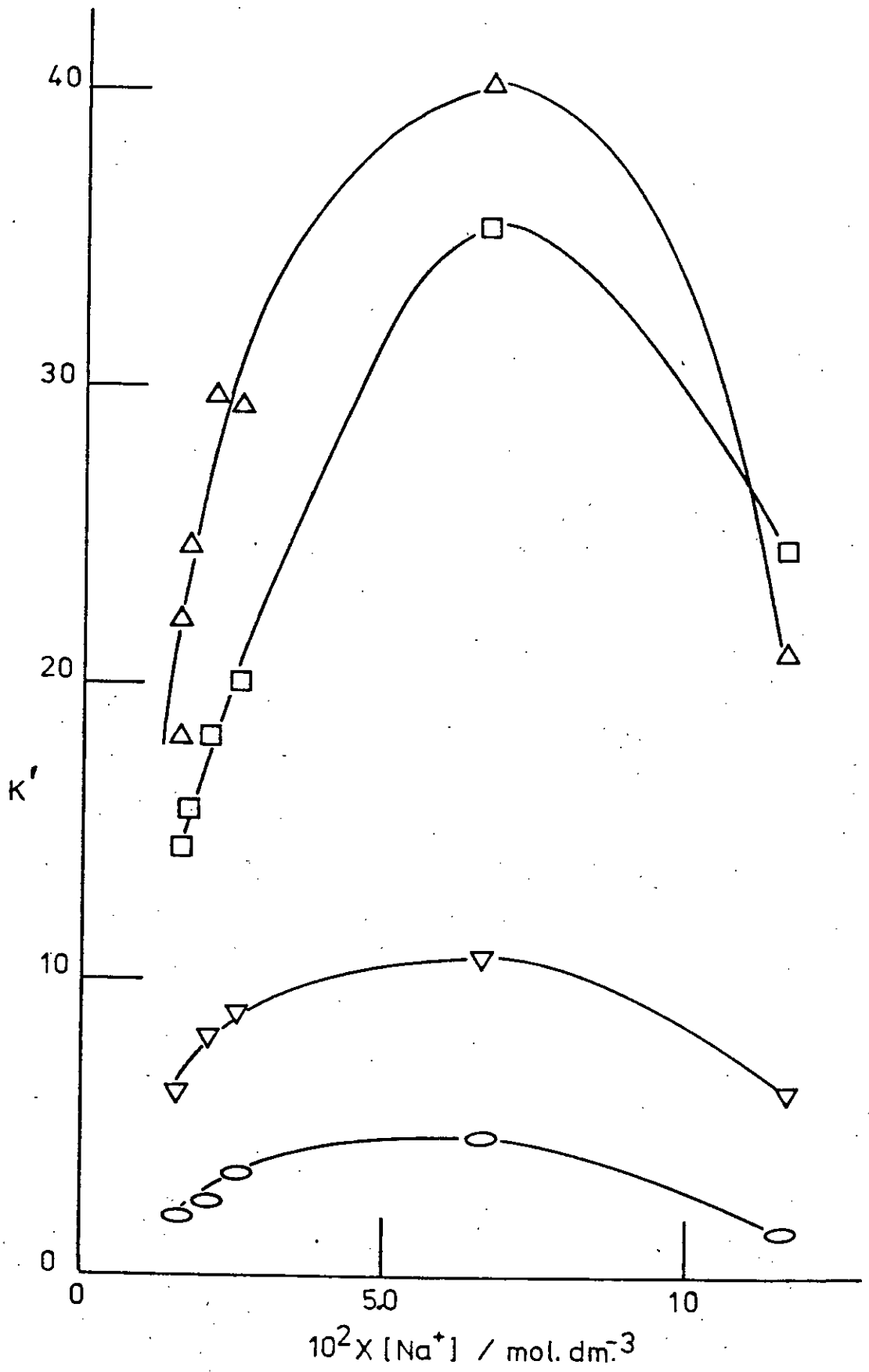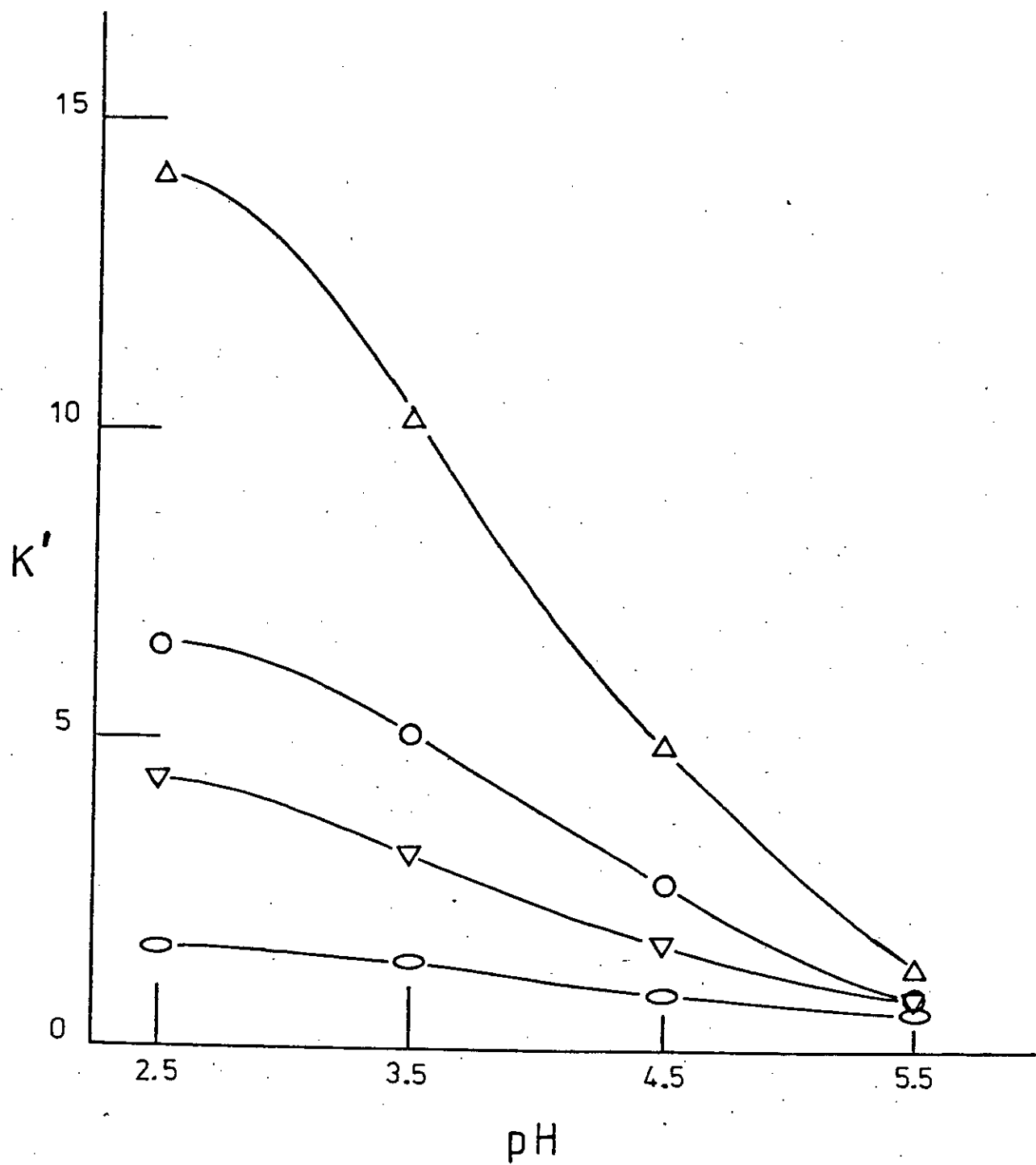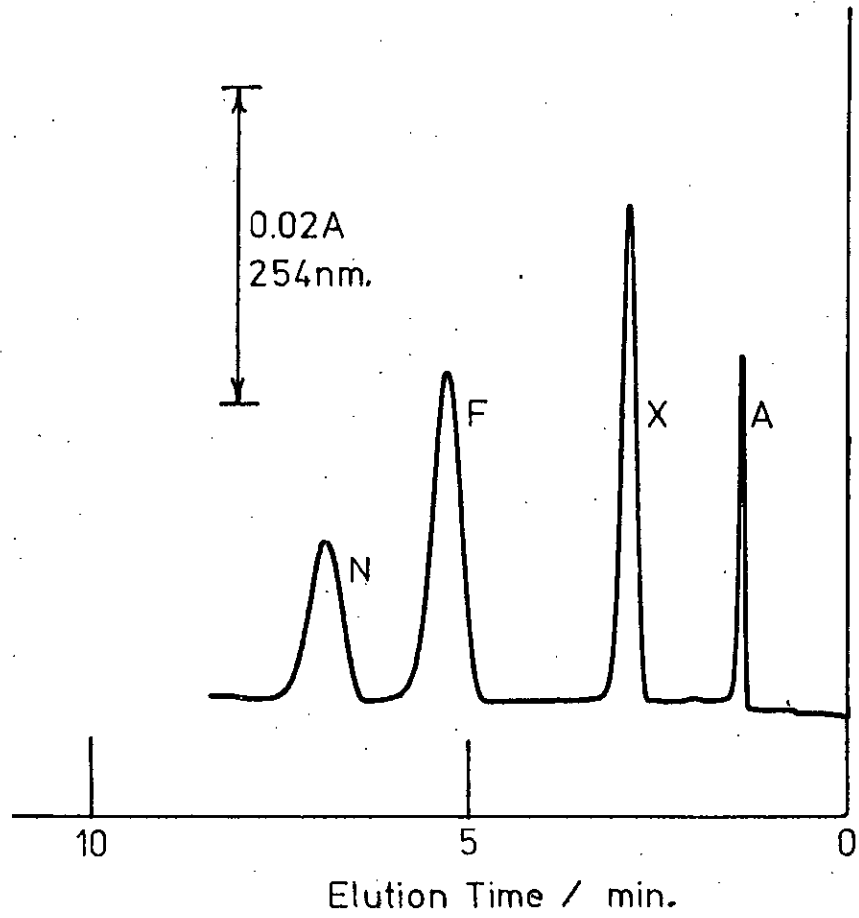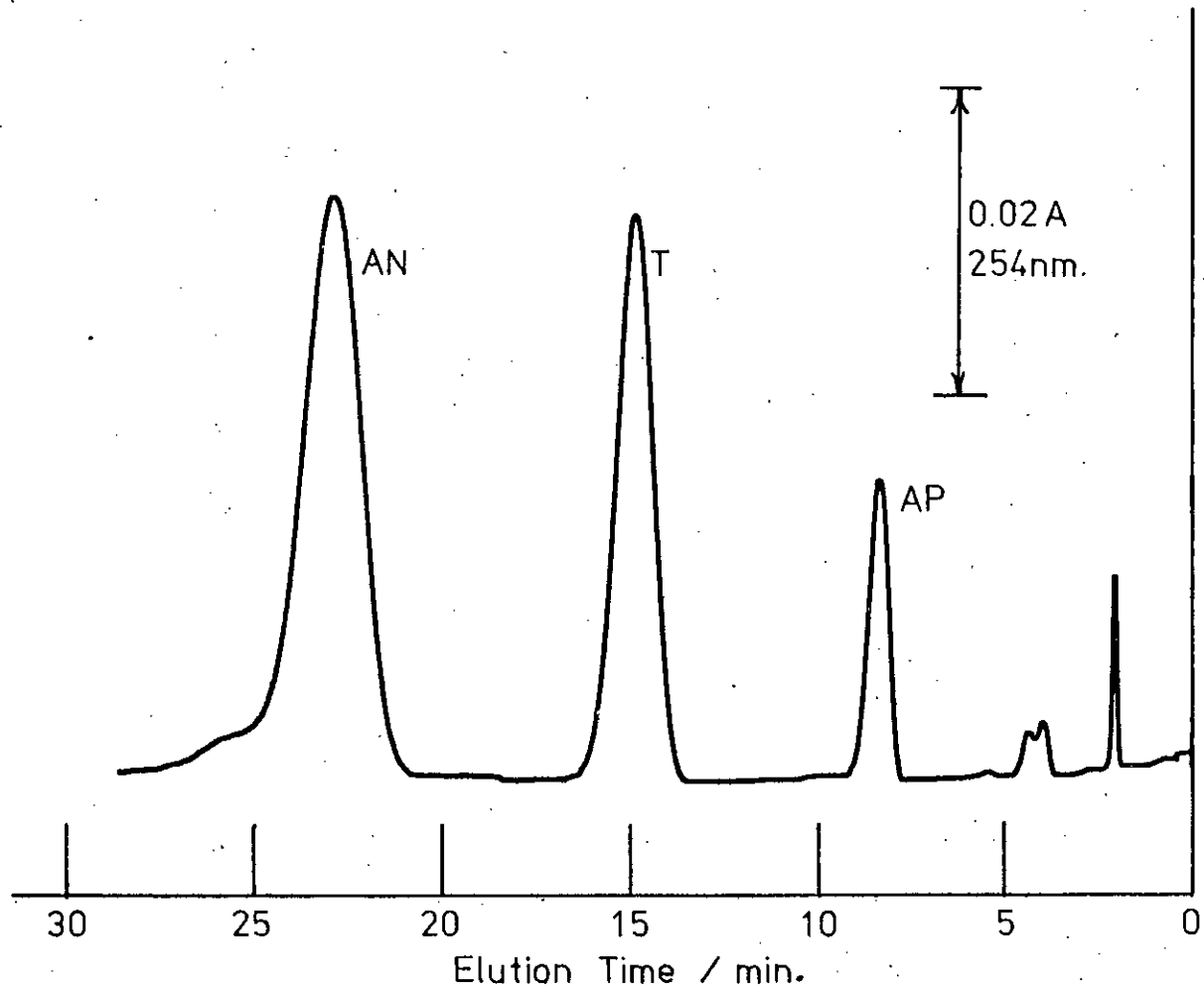    tryptophan methyl ester, and 1-amino-1-(1-naphthyl) ethane.

Surface Coverage / $\mu mol. m.^{-2}$

0.02A
254nm.

N

F

X

A

10       5       0

Elution Time / min.

0.02 A
254nm.

AN

T

AP

30        25        20        15        10        5        0

Elution Time / min.