Studies of solute retention in HPLC using hydrophobic pairing ions with octadecylsilica.

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STUDIES OF SOLUTE RETENTION IN HPLC USING HYDROPHOBIC PAIRING IONS WITH OCTADECYLSILICA

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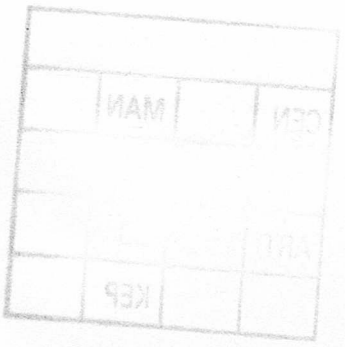

Abstract

Studies of Solute Retention in HPLC Using Hydrophobic Pairing Ions with Octadecylsilica

Cheung-Tak Hung

Following a general historical review of chromatographic development leading to the widespread use of bonded reversed phase ion-pairing chromatography, critical evaluations are made of current theories proposed for the retention mechanisms in such chromatography. In the light of the inadequacies shown by previous theories, experimental work is reported on the adsorption of several widely used anionic and cationic pairing ions on ODS-Hypersil as a typical capped C-18 stationary phase. Using substituted benzoic acids and aromatic amines as test solutes their retention as a function of appropriate pairing ion concentration is studied. For highly adsorbed pairing ions the effect of acetonitrile as organic modifier is also examined. Based on the results obtained, a model of retention involving both ion-exchange and desolvation is proposed and a quantitative relationship formulated between capacity factor and adsorbed pairing ion concentration. The agreement between the predicted capacity factors and those obtained by direct measurement is discussed.

The above ideas have been applied to the separation of the acidic products formed during the autoclaving of D-glucose solutions. By comparison of the chromatographic behaviour of these products with that of standard acids, the acidic products are identified. In the light of results obtained the mechanism of decomposition of D-glucose solutions under autoclaving conditions is discussed.

A systematic study of the chromatography of tricyclic antidepressant drugs is made. In this investigation it is shown that pairing ion alone is inadequate for resolution in certain cases. Optimum separations are shown to be obtained by the inclusion of organic basic counter ions in addition to conventional pairing ion. The results of a systematic study of different counter ions are shown and interpreted as an extension of the ion-exchange-desolvation ideas previously suggested.

The general applicability of organic counter ion ideas is demonstrated for the antimalarial drug proguanil and its metabolites. An improved method of serum determination of these compounds is suggested.

A discussion of the difficulties in attempting to relate chromatographic behaviour using bonded reversed phase silica to the chemical structure of the solute is made utilising experimental data from the literature.

GENERAL SUMMARY

Current theories on the mechanism of retention of ionic solutes in ion-pair chromatography using bonded reversed phase have been critically reviewed and an approach combining ion-pairing and ion-exchange chromatography is suggested.

The isotherms of several commonly used cationic and anionic pairing ions on ODS-Hypersil from pure water have been measured over large ranges in aqueous pairing ion concentration. The effect of organic modifier on the isotherms of cetrimide and sodium laurylsulphate has also been determined using acetonitrile as the organic modifier. The chromatographic behaviour of several acidic and basic solutes in presence of appropriate charged pairing ion for the same chromatographic system is reported over a similar range of pairing ion concentrations.

The results obtained are found not to support the current models used to explain the alteration of retention by pairing ion. The suggested combined ion-pairing-ion-exchange model is found also to be unsatisfactory. An alternative model, based on a combination of ion-exchange and desolvation processes is advanced in which account is taken of the alteration in C-18 surface area available for desolvation due to adsorption of pairing ion. On the basis of this mechanism an equation relating the capacity factor of an ionic solute to the adsorbed concentration of oppositely charged pairing ion and mobile phase counter ion has been derived.

The calculated values of capacity factors of the various solutes studied using data obtained from the isotherm measurements are shown to be in good agreement with those obtained by chromatographic measurement.

The retention behaviour of an uncharged solute 5-hydroxymethyl-furfuraldehyde has also been examined as a function of adsorbed pairing ion concentration for all pairing ions. While the derived equation predicts the observed decrease in capacity factor of such a solute, the equation appears to underestimate the effect produced by adsorption of pairing ion.

This discrepancy is believed to be a result of undertainty as to the nature of the surface structure of the C-18 adsorbent.

The ion pairing ideas developed, in particular, choice of pairing ion and its mobile phase concentration have been applied to the identification of the products produced during the decomposition of D-glucose solutions under autoclaving conditions. In addition to the well known major product of decomposition, 5-hydroxymethylfurfuraldehyde, several intermediate products and two adicic products have been detected chromatographically. The retention behaviour of the two acids is shown to be characteristic of that observed for the previously studied carboxylic acids and also to conform to the established behaviour of acidic species when alteration of pH is utilised to produce ionic suppression.

The retention behaviour of the acids produced on degradation of D-glucose as a function of pairing ion concentration has been compared with that of previously suggested acidic degradation products. It is shown that the variation in capacity factor of such a solute with mobile phase pairing ion concentration, while being general in type i.e., passing through a maximum, is unique to any particular acid in terms of the maximum magnitude of the capacity factor obtained. On the basis of such behaviour the acidic products are identified as 5-hydroxymethylfuroic acid and furan-2, 5-dicarboxylic acid. These identities have been confirmed by the correspondence of their ultraviolet spectra with standard samples of these compounds using stopped flow scanning methods. On the basis of these identifications a mechanism of decomposition of D-glucose to acidic products is suggested.

The problem of resolution and quantitation of the tricyclic antidepressant group of drugs has been investigated utilising the ion pairing technique. In particular it was intended to examine the reported improvement in chromatography resulting from the inclusion of organic amines in

the solvent systems for separation of such basic compounds.

It is shown that while a degree of separation can be obtained among the members of the set of tricyclic antidepressant drugs employed using conventional ion pairing methods, that is adjustment of the concentrations of pairing ion, counter ion and organic modifier at an appropriate pH, inadequate resolution is obtained between clinically important pairs. The effect of adding organic amines of different hydrophobicities has been examined. It is shown that the general effect is that of an organic counter ion that is, increase in organic amine concentration produces a decrease in solute retention. The linear relationship between reciprocal of counter ion concentration and capacity factor is not observed.

It is shown, however, that while no increase in efficiency is obtained as a result of such added organic amine in the case of tricyclic antidepressants, a marked alteration in selectivity is obtained and complete resolution of the previously unresolved pairs of compounds can be obtained as a result of including amines.

The results obtained are interpreted on the basis of the previously proposed ion-exchange-desolvation model of ion pairing and an optimised chromatographic system is suggested for the separation and quantitation of the clinically relevant solute pairs in this drug class. The effect of the different variables affecting such separations including organic counter ion concentration is discussed.

The ideas concerning the importance of organic counter ion in the chromatography of basic drugs is further demonstrated in the systematic optimisation of separation of the antimalarial proguanil from its metabolites. An optimised chromatographic system is reported which improves that currently in the literature. An extraction method is suggested which is shown to produce increases in sensitivity over the direct injection method previously employed for serum sample measurement.

The above findings concerning the manipulation of retention of solutes by alteration of the mobile phase composition is unique to any given solute. In order to predict retention or elution order of a series of solutes a knowledge of the relevant physical properties determining retention would appear to be important.

The current literature on the field of quantitative structure retention relationships is critically reviewed. The current approach of using a single parameter to describe the hydrophobicity of a solute is considered to oversimplify retention processes.

The apparently good linear correlations observed between capacity factor and derived hydrophobicity indexes are demonstrated not to be observed when compounds of widely differing chemical structures are examined.

It is also demonstrated that, even for similar compounds which are ionic in nature, poor correlation is obtained between capacity factor and hydrophobicity index. The limitations of this approach are discussed in the light of factors such as the size and shape of solute molecules and their relationship with the steric structure of the widely used C-18 stationary phases.

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DECLARATION

All the experimental work described in this thesis was carried by C. T. Hung in the laboratories in Robert Gordon's Institute of Technology, Aberdeen in collaboration with Department of Chemical Pathology, University of Aberdeen, Medical School, Foresterhill, Aberdeen and as acknowledged.

It has not been accepted in substance or concurrently submitted in candidature for any other degree.

Candidate

Director of Studies

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Abbreviations used in the text.

AD	L-Adrenaline
AN	Aniline
BA	Benzoic acid
BRPHPLC	Bonded reversed phase high pressure liquid chromatography
BRPIPC	Bonded reversed phase ion-pair chromatography
BZ	Benzylamine
CAMS	dl-10-Camphorsulphonic acid
CETA	Cetrimide
DMA	Dimethylamine
2,4DMBA	2, 4-Dimethylbenzoic acid
DOP	Dopamine
4EBA	4-Ethylbenzoic acid
FDA	Furan-2, 5-dicarboxylic acid
HETP	Height equivalent to a theorectical plate
5HMF	5-Hydroxymethlfurfuraldehyde
5HMFA	5-Hydroxymethylfuroic acid
HPLC	High pressure liquid chromatography
IEDM	Ion-exchange desolvation mechanism
IPC	Ion-pair chromatography
META	Methylammonium
NA	Naphthoic acid
OCTS	1-Octanesulphonic acid
PANS	1-Pentanesulphonic acid
PPLA	Propylamine
QSAR	Quantitative structure activity relationship
QSRR	Quantitative structure retention relationship
RPHPLC	Reversed phase high pressure liquid chromatography
SLS	Sodium laurylsulphate
TBA	Tetrabutylammonium
TEA	Tetraethylammonium
TMA	Tetramethylammonium
TRIEA	Triethylamine
TRIMA	Trimethylammonium

CHAPTER ONE

INTRODUCTION

1.1 History and development of chromatography

温故知新

—— a chinese quotation, meaning "to study the past is to understand the future".

To appreciate how high pressure or high performance liquid chromatography (HPLC) has become a major technique for the analysis of many varieties of compounds in the fields of pharmacy and biochemistry, environmental analysis and food science, it is necessary to go back to the beginning of the 20th Century when chromatography was first developed.

The details of the development, evolution and diversity of the chromatographic techniques in the past half century have been investigated and commented on by several historians [1,2] and authorities [3-5] in this field. It is the aim of this chapter to present a brief discussion of the contributions to the development of chromatography by these pioneers.

What is "chromatography"?

There are many possible definitions for chromatography. It has been defined somewhat cynically as "the best method for a young would-be-scientist to publish a large number of papers rapidly [6]." Whilst the special committee of the International Union of Pure and Applied Chemistry [7] define chromatography as:

> a method, used primarily for separation of components of a sample, in which the components are distributed between 2 phases, one which is stationary while the other moves. The stationary phase may be solid, or a liquid support on a solid, or a gel. The stationary phase may be packed in column, spread as a layer, or distributed as a film etc.:

in these definitions chromatographic bed is used as general term to denote any of the different forms in which the stationary phase may be used. The mobile phase may be gaseous or liquid.

However, to define chromatography is as difficult to define pornography but we know what it is when it is mentioned [5].

Because chromatography is not restricted to the separation of coloured materials it has lost its original meaning. About 10 years ago the suggestion was made to replace the term "chromatography" by a more appropriate one "sorptography".[8]

"Tswettography" [9,10] has also been proposed as a replacement of chromatography as a tribute to the gifted Italo-Russian botanist M. S. Tswett who discovered chromatography as a separation technique in 1903. It is unlikely, however, that any term other than chromatography will now find general acceptance.

Although chromatography was used by Runge [11,12] to develop some fascinating coloured pictures in 1850, Goppelsroeder [13] employed similar techniques in his "Capillary Analysis" in 1861 and Day [14] used Fullers earth to fractionate an oil in 1900, it is now beyond dispute that chromatography was the brainchild of M. S. Tswett in 1903 [15]. This is because Tswett was the first to interpret the physical process involved in chromatography and apply them to the separation of plant pigments. The technique he employed is now called liquid-solid (adsorption) chromatography.

Unfortunately, Tswett's publications [15-19] were not accepted by the then famous scientists [20] and this was the reason why chromatography was not used as a major separation technique for nearly 20 years after Tswett's research period (1903-1912). It was not until the work of Khun and Lederer [21-23] in 1931 employing adsorption chromatography to separate carotenoids that people began to realise the importance of this physical

separation technique. Rigorous researches were then carried out by other scientists such as Karrer [24,25], Ruzica, Zechmeister and Cholnoky [26,27]. Subsequently chromatographic techniques spread across Europe and America, leading to what might be termed the "big bang" period of chromatography (1937-1960).

In 1941, chromatography received new momentum through the work of Martin and Synge; the name A. J. P. Martin remained to dominate the development of chromatographic techniques for the next two decades. Martin, together with Synge, invented normal phase liquid-liquid partion chromatography in 1941 [28]. In their research they used water which was adsorbed on silica gel as stationary phase and chloroform with 0.5% alcohol as mobile phase to achieve the separation of several acetylated amino acids. Three years later, together with Consden and Gordon, Martin introduced paper chromatography [29] which brought revolution to the field of biochemical analysis. Ten years later, Martin and James [30] built the first gas-liquid chromatograph which proved its potential as Synge and Martin had predicted in 1941. That Martin and Synge were awarded a Nobel Prize in 1952, is understandable.

Tiselius, another Nobel Laureate of 1948, also contributed enormously to the development of chromatographic techniques. Tiselius, who had already invented electrophoresis in 1937, systematised liquid absorption chromatography by pointing out three different methods of chromatographic analysis during 1940-1943 [31-33]. These were frontal analysis, elution analysis and displacement analysis. In 1952, Tiselius introduced the gradient elution technique [34], which has greatly improved the chromatographic separation of compounds of widely different chemical structure and significantly increased the peak capacity. Later gel-permeation chromatography was also developed in Tiselius laboratory by Porath and Flodin [35].

Thin layer chromatography, which is still a major technique in the identification of unknown compounds in the field of toxicology and

pharmacognosy, was invented by Izmailov and Shraiber in 1938 [36]. They first called this technique "spot chromatography". However, this technique later received its popularity mainly due to the work of Stahl during 1956-1958 [37,38].

Inorganic ion separation has been made easier since the invention of ion-exchange chromatography, which was the idea of Taylor and Urey [39] in the late thirties. The potential of ion-exchange chromatography was best demonstrated in the identification of elements 99 and 100 from the fall-out of a nuclear explosion in the Pacific Ocean in 1952. In this project, there were only about 200 atoms contained in the sample [40].

Without a sound theoretical basis, chromatography in the early forties remained an art rather than a scientific analytical technique. The first quantitative model which allowed the prediction of the rate of development of the bands in a given chromatogram was published by Wilson in 1940 [42]. In his quantitative model, the chromatographic process was considered as a set of stages each representing a complete equilibrium. In addition it was assumed that the adsorption isotherm of the solute was linear and the effect of solute diffusion was ignored.

The effects of various parameters involved in the complex chromatographic process were simplified by Martin and Synge [28]. They introduced the theoretical plate concept, which had already been proposed by Peters [43] and which was widely used in the distillation industry, as a measure of column efficiency. Although the term height equivalent to a theoretical plate (HETP) has lost its original meaning, in retrospect such ideas did make the quantitative treatment of the chromatographic process much easier.

The theory of chromatography was then intensively studied by De Vault in 1943 [44] and Thomas in 1944-1948 [45,46], both of whom attempted to linearise the equation of chromatography so that non-linear isotherms and non-equilibrium conditions could be treated.

However, it was only after the invention of gas-liquid chromatography (GLC) which could provide retention and dispersion data accurately, that HETP of a column could be related quantitatively to various major parameters. Van Deemter et al. in 1956 combined all the ideas [28,44-47] previously developed and expressed HETP as a function of particle diameter of the stationary phase, solute diffusivity and flow rate of the mobile phase. Since then the famous "Van Deemter plot" has provided invaluable help in optimisation of column conditions. This relationship between HETP and mobile phase flow rate has subsequently modified by Giddings [48] and Knox [49] to use reduced variables and the corresponding result is known as the "Knox plot". The kinetic aspects involved in chromatography have been critically reviewed by Giddings [50].

1.2 High pressure liquid chromatography (HPLC)

While gas-liquid-chromatography was developed as a technique largely on empirical grounds, modern HPLC is based on the theoretical investigations outlined above. Although it was pointed out by Martin and Synge in 1941 [28] that small particle size and high pressure differences across the length of the column were required to produce high efficiency column liquid chromatography, the technology was then unavailable to fulfil these criteria. After the invention of GLC, liquid chromatography was practically ignored by most chromatographers. Hamilton [41] applied HPLC in ion-exchange columns to achieve separation of several amino acids in 1960. His work, however, was specialised in amino acid analysis and as a result made little general impact. It was also shown theoretically by Giddings [51,52] that liquid-chromatography, because of its low diffusivities, should have 100-1000 fold advantage in maximum plates number over GLC. It was not until 1967 that Huber et al. [53] and Horvath et al. [54] demonstrated that liquid chromatography performance based on speed and efficiency was

comparable to that of GLC. Their results were achieved when small particle size stationary phases and large pressure differences were employed as predicted by theory. The success attracted the interest of not only chromatographers but also equipment manufacturers. The results obtained by this new technique coupled with commercial exploitation led to a very rapid expansion of effort both in the areas of equipment technology and in application of the technique.

While very many scientists have contributed to the field of HPLC G. Guichon, I. Halasz, C. Horvath, J. F. K. Huber, B. L. Karger, J. J. Kirkland, J. H. Knox, R. E. Majors, R. P. W. Scott, L. R. Snyder and K. K. Unger are among the most notable. As a result of their effort HPLC has now superseded its counterpart GLC for much chemical analysis.

Technology is approaching the theoretical limit for packed columns and future improvements in resolution and peak capacity are likely to be as a result of using capillary columns. The detection systems employed are far from ideal and the mass spectrometer offers the best hope for completely non-specific detection at the sensitivities required. It is likely that future advances in HPLC performance will be as a result of combining capillary columns with mass spectroscopic detection [134].

1.2.1 Reversed-phase-HPLC (RPHPLC)

Reversed-phase liquid chromatography was first suggested by Boscott [55] in 1947 and successfully applied by Boldingh [56] in 1948 to separate methyl esters of long chain fatty acids. Filter paper impregnated with vulcanized rubber powder was used with aqueous methanol and acetone as solvent. The name "reversed-phase" chromatography was first coined by Howard and Martin [57] in 1950. These workers used liquid paraffin coated on chemically treated kieselguhr as the stationary phase and aqueous methanol as solvent to separate C12-C18 fatty acids.

When HPLC was first applied in the reversed phase mode, the non-polar stationary phase were coated on to some inert solid absorbents by the solvent evarporation methods used in gas chromatography. Such high percentage loadings (10-20%), on porous supports were difficult to pack, with low efficiency and severe peak tailing due to the poor mass transfer. Attempts to use the pellicular supports developed by Kirkland [58] for normal phase chromatography significantly improved column efficiency. These supports consisting of a non-porous core surrounded by a porous shell offered lower liquid loadings (1% w/w) and thus improved mass transfer. Kirkland in attempting to improve such mechanically held liquid phases also modified the stationary phase surface before liquid coating [66].

However, such pellicular stationary phases suffered from the major disadvantages of decreased sample and peak capacities. Although mechanically held reversed-liquid-liquid HPLC can give satisfactory results in many analysis, it suffers from a number of disadvantages. Time is required for equilibration between the stationary and mobile phases, a presaturation column is necessary, reproducibility is poor and gradient elution techniques are forbidden.

The above problems of the mechanically held stationary phase in RPHPLC can be eliminated if the stationary phase is chemically bonded to the inert supports. Silica, because of its established technology in terms of particle size and uniformity, is almost exclusively used as such a support. The first stationary phase having an organic group chemically bonded to the surgace of the silica was developed by Halasz and Sebastian [59] in 1969. The organic molecule 3-hydroxypropionitrile was esterfied with the silanol group on the surface of the silica to give a stationary phase $\equiv SiO(CH_2)_3CN$, with the polar cyano group freely exposed into the mobile phase. The major disadvantages of such silicate bonded phases is that they are thermally unstable and can easily be hydrolised in aqueous mobile phases.

Improvement in the bonding techniques led to the development of numerous bonded reversed-phases which appeared in the early 1970s. Organic molecules can be bonded to the surface of the silica in three different ways:

(1) Formation of aminosilanes (≡ Si-N-C) by treatment of silica chloride with amines [60] results in reasonable stability in aqueous systems.

(2) Siloxane bonded phases (≡ Si-O-Si), introduced by Kirkland [61] and Majors [62] provide sufficient stability under conditions used in the most chromatographic analysis.

(3) The silica-carbon (≡ Si-C) bonded phases developed by Locke [63] are both thermally and solvolytically stable.

The stability of chemically bonded phases increases from (≡ Si-N-C), through (≡ Si-O-Si) to (≡ Si-C) bonding. The difficulty in synthesis and reproducibility also increases in the same order. The most stable (≡ Si-C) bonded phases are unavailable commercially since the salt that is formed during the manufacturing process is very difficult to remove. All the chemically bonded reversed phases now available commercially are siloxane bonded [64].

The present day very extensive use of reversed-phase HPLC is due to many advances that have taken place in recent years. These include the reduction of stationary phase particle size to 5-10 μm [65] together with the use of totally porous particles. The improvement of bonding techniques has increased reproducibility of performance. Slurry packing methods which are necessary for such small particle size materials are now well established [66,67] and column reproducibility is readily attained. Of all the bonded stationary phases employed by chromatographers octyl-(C8) and octadecyl-(C-18) silica are by far the most popular. It

has been estimated over 80% of HPLC applications employ C-8 or C-18 silicas [68].

Though the advent of RPHPLC technology in the past several years has resulted in a tremendous growth in the popularity and applications of reversed bonded phases, the exact retention mechanism involved in this technique remain uncertain. One approach to understanding the process of retention has been the solvophovic theory proposed by Horvath et al. [71,72]. This describes retention of hydrophobic solutes as being due to compulsive adsorption due to the expulsion of the solute out of the aqueous mobile phase. Extensive investigations using different stationary phases [70,115] have produced evidence to support this picture.

However, any uncertainty in the understanding of the retention mechanism of RPHPLC has not reduced its convenience and versitility. A single column can suffice for most separations including non-polar, polar and ionic solutes [73]. In the case of strongly acidic and basic compounds, use of conventional RPHPLC technique to achieve retention and separation will not be practical, because of the requirement of extreme pH values to suppress ionization of the solutes. When the pH value is beyond the range of 2-8, damage to the n-alkyl siliceous supports occurs [74]. The alternative approach to retention of ionogenic solutes is that proposed by Horvath [72] involving high electrolyte concentration in almost purely aqueous solvents. This results in good efficiency for some solutes but tends to produce severe band broadening when solute molecules are large. Twitchett, in assessing the usefulness of RPHPLC indicated [75] that while most neutral and acidic compounds could be analysed by this method, difficulties arose with even weakly basic solutes. Classical ion exchange methods [76], until recently were extensively used for chromatography of strong acids and bases. One of the major advances in HPLC recently was the inception of ion pair chromatography to achieve

retention and separation of ionic and ionizable species [77].

1.3 Ion-pair chromatography (IPC)

There are 3 kinds of ion-pair liquid chromatography: normal phase ion-pair partition chromatography, reversed phase liquid-liquid ion-pair partition chromatography and chemically bonded reversed phase ion-pair chromatography (BRPIPC). In normal phase ion-pair liquid-liquid partition chromatography, a aqueous stationary phase which contains a hydrophilic pairing ion e.g. perchlorate or phosphate buffer is mechanically held on the inert support silica gel. The mobile phase is an organic solvent which is immiscible with water. It is suggested that the solute elutes as an ion-pair in the mobile phase. Normal phase ion-pair partition chromatography has been applied in the separation of several biogenic amines [78] and tricyclic antidepressants [79] with excellent selectivity and efficiency observed. However, such a technique is difficlut to perform and is now practically unused.

Reversed-phase liquid-liquid partition chromatography involves the addition of low concentrations of hydrophobic pairing ion to augment the retention and separation of the ionised solutes. Here, it is suggested, the ionised solute is dissolved into a mechanically held organic liquid stationary phase as a neutral ion-pair complex formed between the solute and the pairing ion. Such a process was demonstrated by Wahlund and Groningen [80]. The above mechanism was based on the ideas of ion-pair extraction in which ionised solutes can be extracted into an organic phase in presence of the oppositely charged hydrophobic pairing ions. The batch extraction methods of ionised solutes were extensively studied and experimentally investigated by Schill [81,82] and Huguchi [83].

With the advantages of bonded phases well established it was logical that ion-pair methods should be applied to such systems. Chemically bonded reversed-phase ion-pair chromatography without the addition of a liquid

phase is presently the most generally used approach in ion-pair chromatography, although liquid-liquid partition-(IPC) can be still carried out using chemically bonded supports if low concentrations of higher alcohols are added to the mobile phases. Such methods, however, are limited to a few workers [84,85].

The use of BRPIPC in the separation of various ionogenic solutes by addition of hydrophobic pairing ion with long alkyl chains to the mobile phases was pioneered by Haney et al. [86,87], Waters Associates Inc. [88] and Knox et al. [89,90] in the mid 1970 s. Results obtained using this development produced controversy concerning the nature of the process responsible for retention. Haney et al. [86] argued that, the mechanism of separation in BRPIPC appeared to be the reversible formation of ion-pairs within the chromatographic system, and separation of the constituents was on the basis of differences in the hydrophobicity of the ion pairs so formed. However, the argument of reversible ion-pair formation is vague and makes the attempt to interpret the retention and separation mechanisms quantitively very difficult. Experiments by Knox and Laird [89] showed that the hydrophobic pairing ion which was initially present in the mobile phase was adsorbed onto the hydrophobic organic support. Thus they interpreted the retention mechanism in BRPIPC as a dynamic ion-exchange process. Such a concept was further heralded by the communication of Kissinger [91] who urged that the retention and separation mechanism in BRPIPC was far from being well understood [77]. He believed that the dynamic ion-exchange idea was the more likely.

It has been demonstrated by very many workers [90,92-103] that, over a wide range of pairing ion concentration in the mobile phase, the capacity factors of ionic solutes show a complex dependence on pairing ion concentration. The capacity factor may reach a plateau and in many cases pass through a maximum. Such evidence indicates that the retention process

during BRPIPC is considerably more involved than those described by either ion-pairing or ion-exchange concepts. This may be the reason for the proliferation of nomenclature applied to this separation technique. BRPIPC has also been referred to as "paired-ion chromatography" [88], "soap chromatography" [89], "solvent generated dynamic ion-exchange chromatography" [104], "detergent based ion-exchange chromatography" [104], "surfactant chromatography" [105], "haeteric chromatography" [93], "solvophobic-ion chromatography" [107], "ionic-interaction chromatography" [108] and "mixed dynamic ion-exchange-ion-pairing chromatography" [109]. Such a variety of nomenclature only indicates the uncertainty as to the mode of action which exists in BRPIPC.

Although the process occurring in BRPIPC have been described by such diverse names, only two clearly distinguished mechanisms have been suggested and treated quantitatively. These are the ion-pairing and ion-exchange mechanisms. The ion-pairing mechanism in BRPIPC assumes the formation of an ion-pair complex between the ionic solute species and the pairing ion of opposite charge in the mobile phase prior to its adsorption onto the stationary phase [93,106,109]. However, it has also been suggested that the ion-pair complex instead of being formed in the mobile phase, occurs on the surface of the organic support [100,110]. The reason suggested is that the dielectric constant on the surface of the organic stationary phase is much lower than that in the polar mobile phase thus facilitating ion-pair complex formation. Both of these ion-pair concepts stress the importance of ion-pair formation in the retention of ionic solutes, but the models derived on the basis of the different approaches are quite dissimilar.

The ion-exchange mechanism in BRPIPC differs from the ion-pairing concept in that the hydrophobic pairing ion is adsorbed onto the organic stationary phase and causes the column to behave as an ion-exchanger [89]. The details of each of the mechanisms having hitherto been proposed will be discussed in detail in the next chapter.

It is obvious that the above different approaches to solute retention will result in different design strategies for separation of ionic solutes using BRPIPC. If ion-pairing is the mechanism, the concentration of the pairing ion in the mobile phase is the governing factor in the chromatography. However, if the ion-exchange idea prevails, the pairing ion concentration on the organic support is of major importance in the retention and separation process. So far, BRPIPC remains an art, solely because the retention mechanism involved is not well understood and considerable trial and error is required for successful chromatography. It would be advantageous if the retention process could be clarified so that operating procedures could be systematised.

CHAPTER TWO

A STUDY OF THE RETENTION MECHANISMS OF IONIC SOLUTES IN REVERSED-PHASE-ION-PAIR-HPLC

2.1 Nomenclature

Due to the different avenues by which liquid chromatography has developed together with the fact that very often chromatography was used as a means to an end by workers in diverse disciplines, no standard nomenclature for chromatographic terms has yet been established. The expansion of the chromatographic method to its present widespread use has resulted in attempts to define general terms and symbols. Such nomenclature systems are defined by the American Society for Testing and Methods (ASTM) [116] and by the International Union of Pure and Applied Chemistry (IUPAC) [7]. These systems while not identical are similar and a review on terminology has been given by Ettre [117].

However, in ion-pair liquid chromatography especially when reversed bonded stationary phases are employed, terminology remains confusing [105]. For convenience of notation a list is given below of the principal terms used in this work together with their symbols and meanings.

Pairing ion, ($P^{\pm}$), is the hydrophobic ion of the appropriate charge added to the aqueous mobile phase which complexes with an inogenic solute species ($S^{\pm}$) of the opposite charge.

Counter ion, ($C^{\mp}$), is the inorganic or organic ion which possesses the same charge as the ionic or ionised solute ($S^{\mp}$) and is usually added together with the pairing ion as ($P^{+}C^{-}$) or ($P^{-}C^{+}$), or with the ionic solute as ($S^{+}C^{-}$) or ($S^{-}C^{+}$), or as one ion of the buffer salt in the aqueous mobile phase.

Capacity factor, (k'), is a retention parameter defined as,

$$k' = \frac{V_r - V_m}{V_m} = \frac{t_r - t_o}{t_o}$$

V_r and V_m are the volumes of the mobile phase required to elute the solute and an unretained test solute respectively. Alternatively t_r and t_o

representing retention times, can be substituted for the corresponding volume terms.

Relative retention, (α), of a pair of solutes is defined as,

$$\alpha = \frac{t_{r2} - t_o}{t_{r1} - t_o} = \frac{k'_2}{k'_1}$$

Where k'_2 is greater than k'_1.

Efficiency of a column, (N), is defined as, $N = 5.54 \times (t_r/w)^2$, where w is the peak width at half peak height.

Phase ratio, (ϕ), is defined as $\phi = As/V_m$, where As is the total surface area of the organic stationary phase and V_m is the void volume of the column.

Distribution coefficient, (Ds), is defined as the ratio of the concentration of the sample adsorbed and the sample in the mobile phase at equilibrium i.e.,

$$Ds = [S\overline{+}]org/[S\overline{+}]aq.$$

The subscripts org and aq denote the organic stationary phase and aqueous mobile phase respectively.

2.2 Theoretical

Reversed phase liquid-liquid partition chromatography was initially studied by Wahlund et al.[80], but was first treated quantitatively by Eksborg and Schill[118]. The basis of this concept was derived from the batch extraction process[81,82]. It has been generally agreed that such classical ideas appeared to be the basis of the retention mechanism operating in normal-phase partition chromatography[91], and this technique will not be considered further. However, in the reversed-phase mode the retention mechanism is still a matter of some controversy. Several models have been proposed to describe the process thought to

occur during BRPIPC. These differ both in the types of processes producing retention and in the results of theoretical treatments attempting to relate capacity factors with concentration of pairing ions. The differing approaches to the understanding of the mechanism of retention in BRPIPC will be discussed individually below.

2.2.1 Ion-pair chromatography assuming the formation of an ion-pair on the organic stationary phase only

This model was first proposed by Schill et al.[118] and was the motivation in the development of IPC. It was argued that, the dielectric constant in the diffusion layer of the organic support is much lower than that in the mobile phase, and that this would facilitate ion-pair formation in this region[105]. In support of this the work of Bjerrum[119] is often quoted. This early work demonstrated that even the inorganic salt sodium chloride would fail to behave as a strong electrolyte in liquid ammonia. Thus the ion pair formed in IPC will behave as a polar organic molecule and will therefore be preferentially adsorbed into the organic stationary phase.

A model based on the above assumption was represented as,

$$(S^{\mp})aq + (P^{\pm})aq \underset{}{\overset{K_{eq}}{\rightleftharpoons}} (P^{\pm}S^{\mp})org \qquad -(1)$$

and was treated mathematically as follows:

$$K_{eq} = [P^{\pm}\ S^{\mp}]org\ /\ [P^{\pm}]aq\ [S^{\mp}]aq \qquad -(2)$$

$$Ds^{\mp} = [P^{\pm}\ S^{\mp}]org\ /\ [S^{\mp}]aq \qquad -(3)$$

$$\text{and } k' = \phi\ .\ K_{eq}.[P^{\pm}]aq \qquad -(4)$$

As shown in equation (4), the capacity factor (k') of a ionic solute has a linear relationship with the concentration of pairing ion in the mobile phase. This linear dependence has been demonstrated by numerous workers[120-123]. In this model, it is assumed that there is

insignificant adsorption of pairing ion onto the stationary phase and the phase ratio (ϕ), can be regarded as constant and unaffected by the variation of mobile phase pairing ion concentration.

The above assumptions have been shown to be questionable by evidence that hydrophobic pairing ions are indeed adsorbed onto the n-alkyl modified silica to various extents [89,95,98,124]. Also the capacity factor of an ionic solute has been observed to show a complex dependence on the mobile phase concentrations [92-103]. In the light of these facts the above ion-pair model has recently been modified [100,110,135].

In the modified model, the authors have considered that adsorption of pairing ion may occur on particular sites and postulate two types of adsorption sites. It is suggested that one such site has a high efficiency for the solute-pairing ion complex and the other for the weak complex involving the pairing ion and its associated inorganic counter ion. The amended model results in a more complex relationship between capacity factor and mobile phase pairing ion concentration than that given by equation (4). It approximates to the non-linear variation observed experimentally.

This model depends to a large extent on the assumption of two particular adsorption sites which appears inconsistent with current ideas on the nature of the C-18 surface [151]. In addition it is not clear in the derivation whether account is taken of the implied change in the phase ratio resulting from adsorption of the pairing ion-counter ion complex. The formation of ion pairs in the mobile phase suggested by other models has been rejected on the basis of experimental measurements.

2.2.2 Ion-pair chromatography assuming the formation of an ion-pair in the mobile phase followed by subsequent transfer onto the hydrophobic stationary phase

While the above model considers that ion-pair complexes could only

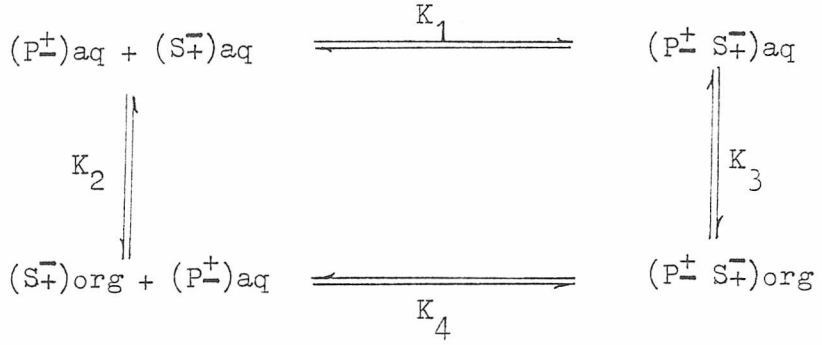
exist in solvents of low dielectric constants [119], the work of Diamond [125] has suggested the possibility of ion-pair formation between two hydrophobic ionic substances in aqueous solvents produced by the so-called hydrophobic force. In a more correct sense, the formation of an ion-pair complex of two hydrophobic ions will decrease the disturbance of the solvent structure. As a consequence, the increase in entropy of the system, thermodynamically favours ion-pair formation even though the dielectric constant in the aqueous mobile phase is high.

Such effects have been studied extensively and a hydrophobic theory has been formulated by Sinanoglu [126] and Sinanoglu and Abdulnar [127]. Horvath et al. [71,72] have recently proposed that retention of non-polar solutes and ionic solutes with hydrophobic substituents is achieved in bonded reversed phase liquid chromatography by such hydrophobic effects and a "solvophobic theory" of chromatography has been developed.

According to the solvophobic theory [71,72], the association of the hydrophobic solute with the C-8 or C-18 surface of the support is a weak reversible compulsive adsorption, resulting from the solute being squeezed out of the mobile phase. In the light of such a solvophobic effect it can be envisaged that both the hydrophobic pairing ion and ionised solute are adsorbed onto the hydrophobic support to some extent [71,72,93]. However, in the presence of hydrophobic pairing ion, the formation of ion-pairs between the solute and the pairing ion is suggested to occur mainly in the mobile phase prior to being transferred onto the surface of the organic support.

Recently this model has been treated mathematically [93]. This treatment takes into consideration the adsorption of the inogenic solute and pairing ion onto the organic stationary phase. The possible equilibra involved are represented as in Scheme (1).

Scheme (1)



and mathematical treatment of the equilibra represented results in the following equations,

$$k' = \phi \cdot \left\{ \frac{[P^{\pm}\ S^{\mp}]org + [S^{\mp}]org}{[S^{\mp}]aq + [P^{\pm}\ S^{\mp}]aq} \right\} \qquad -(5)$$

$$[P^{\pm}\ S^{\mp}]org = K_3 \cdot [P^{\pm}\ S^{\mp}]aq$$

$$= K_1 \cdot K_3 \cdot [P^{\pm}]aq \cdot [S^{\mp}]aq \qquad -(6)$$

$$[S^{\mp}]org = K_2 \cdot [S^{\mp}]aq \qquad -(7)$$

$$[P^{\pm}\ S^{\mp}]aq = K_1 \cdot [P^{\pm}]aq \cdot [S^{\mp}]aq \qquad -(8)$$

Substitute equations (6–8) into (5)

$$k' = \phi \cdot \left\{ \frac{K_1 \cdot K_3 \cdot [P^{\pm}]aq \cdot [S^{\mp}]aq + K_2 \cdot [S^{\mp}]aq}{[S^{\mp}]aq + K_1 \cdot [P^{\pm}]aq \cdot [S^{\mp}]aq} \right\}$$

$$= \phi \cdot \left\{ \frac{K_1 \cdot K_3 \cdot [P^{\pm}]aq + K_2}{1 + K_1 \cdot [P^{\pm}]aq} \right\} \qquad -(9)$$

The difference between these two ion–pairing models lies in the dependen of the capacity factor on the mobile phase pairing ion concentration. In the original form of the first model, the k' of the ionised solute species has a linear dependence on the mobile phase pairing ion concentration. In the second model a rectangular hyperbolic dependence is predicted and k' reaches a constant value. That is as the mobile phase

pairing ion concentration increases up to the situation that $K_1 \cdot K_3 \cdot [P^{\pm}]_{aq}$ is much greater than K_2 and $K_1 \cdot [P^{\pm}]_{aq}$ is much greater than 1 in equation (9), this equation becomes,

$$k' = \phi \cdot \left(\frac{K_1 \cdot K_3 \cdot [P^{\pm}]_{aq}}{K_1 \cdot [P^{\pm}]_{aq}} \right)$$

$$= \phi \cdot K_3 \qquad -(10)$$

The rectangular hyperbolic dependence of the k' on the mobile phase pairing ion concentration has been observed by several workers [93,99,101] and has been taken as evidence supporting this hypothesis. Again, in this model, although the authors realised that pairing ion is adsorbed onto the C-8 or C-18 surface, the concentration adsorbed is assumed to be very small and is ignored. The phase ratio is again regarded as being constant.

In the light of increasing evidence of appreciable adsorption of pairing ion, a so-called "mixed ion-exchange-ion-pairing" model has been suggested [109]. This takes into account the ionic interaction between the ion-pair complex and the adsorbed pairing ion on the C-8 or C-18 surface. It is still contended, however, that ion-pair formation in the mobile phase is the factor of major importance in the retention of ionic solutes in BRPIPC. Conflicting evidence has been presented concerning the existence of ion-pair complexes in chromatographic phases [108,128] which thus casts doubt upon the validity of the above models.

2.2.3 Solvent-generated-dynamic-ion-exchange chromatography

It was noticed that in BRPIPC, many void volumes of mobile phase containing the required mobile phase pairing ion concentration had to be passed through a column before chromatographic conditions were stabilised [89,91]. Following such an indication that the pairing ion originally in aqueous mobile phase was adsorbed by the organic stationary phase, an

alternative mechanism based on the in-situ formation of an ion-exchanger was proposed [89]. According to the dynamic ion-exchange mechanism the possible equilibria can be presented as in Scheme (2) [89,123,124]

Scheme (2)

$$(S^{\mp})aq + (P^{\pm}\ C^{\mp})aq \underset{}{\overset{K_1}{\rightleftharpoons}} (P^{\pm}\ S^{\mp})aq + (C^{\mp})aq$$

$$\Big\| K_5$$

$$(S^{\mp})aq + (P^{\pm}\ C^{\mp})org \underset{}{\overset{K_6}{\rightleftharpoons}} (P^{\pm}\ S^{\mp})org + (C^{\mp})aq$$

The equilibria can be mathematically treated as follows:

$$(P^{\pm}\ C^{\mp})aq \underset{}{\overset{K_5}{\rightleftharpoons}} (P^{\pm}\ C^{\mp})org \qquad -(11)$$

$$(S^{\mp})aq + (P^{\pm}\ C^{\mp})org \underset{}{\overset{K_6}{\rightleftharpoons}} (P^{\pm}\ S^{\mp})org + (C^{\mp})aq \qquad -(12)$$

$$K_6 = \frac{[P^{\pm}\ S^{\mp}]org.\ [C^{\mp}]aq}{[S^{\mp}]aq.\ [P^{\pm}\ C^{\mp}]org} \qquad -(13)$$

$$Ds^{\mp} = \frac{[P^{\pm}\ S^{\mp}]org}{[S^{\mp}]aq} \qquad -(14)$$

$$k' = \phi.Ds^{\mp} = \phi.\left\{\frac{[P^{\pm}\ S^{\mp}]org}{[S^{\mp}]aq}\right\} \qquad -(15)$$

$$= \phi.K_6.\left\{\frac{[P^{\pm}\ C^{\mp}]org}{[C^{\mp}]aq}\right\} \qquad -(16)$$

In this model, ion-pair formation in the mobile phase is regarded as insignificant. This result shows that the operative quantity is the concentration of pairing ion present on the stationary phase in contrast to previous results. It also indicates an inverse dependence of k' on counter ion concentration. Although the dynamic-ion-exchange mechanism implies that part of the organic stationary phase will be covered by adsorbed pairing ion, the phase ratio is once again considered as constant. Using an appropriate isotherm, quantitative relationships between solute

capacity factors and mobile phase pairing concentration have also been established [89,99,124]. The inverse dependence of k' on counter ion concentration has further supported the ion-exchange hypothesis [123,129]. Recently the dependence of the capacity factors on adsorbed pairing ion and mobile phase counter ion concentration predicted on the above has been demonstrated, which further supports the ion-exchange idea [99].

2.2.4 An approach combining ion-pairing and ion-exchange chromatography

In an attempt to consider all possible equilibria which could be responsible for retention in BRPIPC Scheme (3) was formulated in the early stages of the present work [154].

Scheme (3)

$$\begin{array}{ccccc} (P^{\pm}_{n}C^{n\mp})org + n(S^{\mp})aq & \xrightleftharpoons{K_5} & (P^{\pm}_{n}C^{n\mp})aq + n(S^{\mp})aq & \xrightleftharpoons{K_1} & n(P^{\pm}S^{\mp})aq + (C^{n\mp})aq \\ \Big\Vert K_6 & & \Big\Vert K_2 & & \Big\Vert K_3 \\ n(P^{\pm}S^{\mp})org + (C^{n\mp})aq & \xrightleftharpoons{K_4} & n(S^{\mp})org + (P^{\pm}_{n}C^{n\mp})aq & \xrightleftharpoons{K_4} & n(P^{\pm}S^{\mp})org + (C^{n\mp})aq \end{array}$$

$(P^{\pm}_{n}C^{n\mp})$ refers to a monovalent pairing ion together with the possibly multivalent (n) counter ion.

In this model it is assumed that the pairing ion present either in the mobile phase or adsorbed on the stationary phase will interact with the ionic solute to different extents. This scheme produces the following retention equilibra:

$$(P^{\pm}_{n}C^{n\mp})aq \xrightleftharpoons{K_5} (P^{\pm}_{n}C^{n\mp})org \qquad -(19)$$

$$(P^{\pm}_{n}C^{n\mp})org + n(S^{\mp})aq \xrightleftharpoons{K_6} n(P^{\pm}S^{\mp})org + (C^{n\mp})aq \qquad -(20$$

$$(S^{\mp})aq \xrightleftharpoons{K_2} (S^{\mp})org \qquad -(21)$$

$$(P^{\pm}_{n}C^{n\mp})aq + n(S^{\mp})aq \underset{}{\overset{K_1}{\rightleftharpoons}} n(P^{\pm}S^{\mp})aq + (C^{n\mp})aq \qquad -(22)$$

$$(P^{\pm}S^{\mp})aq \underset{}{\overset{K_3}{\rightleftharpoons}} (P^{\pm}S^{\mp})org \qquad -(23)$$

Due to the fact that hydrophobic pairing ion is adsorbed by the organic stationary phase to some extent [89,99,100,108,122], the surface of the stationary support will be modified by the adsorbed pairing ion. The polar ion-pair complex formed between the pairing ion and the ionic solute will thus be repelled by the charged stationary phase. It has been demonstrated that k' of nonionic polar solutes are indeed decreased with increasing mobile pairing ion concentration [95,131]. For this reason, it is considered that the chemical equilibrium represented by Equation (23) is unimportant if not prohibited in the retention mechanism in BRPIPC.

The k' of an ionic solute in BRPIPC can be represented as,

$$k' = \phi \cdot \left\{ \frac{[P^{\pm}S^{\mp}]org + [S^{\mp}]org}{[S^{\mp}]aq + [P^{\pm}S^{\mp}]aq} \right\} \qquad -(24)$$

as in Equation (5). To obtain a relationship between k' and pairing ion concentration in mobile and stationary phases, the above equilibria have to be considered.

$$[P^{\pm}S^{\mp}]org = \frac{K_6^{1/n} \cdot [P^{\pm}_{n}C^{n\mp}]^{1/n}org \cdot [S^{\mp}]org}{[C^{n\mp}]^{1/n}aq} \qquad -(25)$$

$$[S^{\mp}]org = K_2 \cdot [S^{\mp}]aq \qquad -(26)$$

$$[P^{\pm}S^{\mp}]aq = \frac{K_1^{1/n} \cdot [P^{\pm}_{n}C^{n\mp}]^{1/n}aq \cdot [S^{\mp}]aq}{[C^{n\mp}]^{1/n}aq} \qquad -(27)$$

and, substituting equations (25), (26) and (27) into (24), we obtain

$$k' = \phi \cdot \left\{ \frac{K_6^{1/n} \cdot [P^{\pm}_{n}C^{n\mp}]^{1/n}org + K_2 \cdot [C^{n\mp}]^{1/n}aq}{K_1^{1/n} \cdot [P^{\pm}_{n}C^{n\mp}]^{1/n}aq + [C^{\mp}]^{1/n}aq} \right\} \qquad -(28)$$

Equation (28) predicts that an increase in adsorbed pairing ion will increase k' of the ionic solute as has been demonstrated elsewhere [89-104]. Provided K_2 is much smaller than 1, equation (28) can also explain the decrease in k' of an ionic solute with increasing counter ion concentration [123,129]. When the surface of the C-18 is saturated with adsorbed pairing ion, any further increase in $\left[P^{+}_{n}C^{n-}_{+}\right]_{aq}$ will act to reduce k'. This decrease has been observed experimentally by numerous workers [89,92-100] and has been accounted for on the basis of micelle formation [88].

As in all previous models, the phase ratio, $\emptyset$, in this approach is regarded as constant, based on the assumption that the area occupied by the adsorbed pairing ion is minimal. A similar treatment to the above has recently been given in the literature [103]. The equilibria are similar as is the resulting equation connecting k' and pairing ion concentration. The Reference [103] is more rigorous in that it included the counter ion of the solute species. This work supports the fundamental assumption in the above derivation that formation of ion pairs in the mobile phase will act to reduce retention of the solute.

An alternative treatment of the above combined approach has also appeared [109] which, however, considers the formation of ion pairs as contributing positively to the retention process.

A phenomenological explanation of retention in BRPIPC has been given by Bidlingmeyer et al [108] and current work [103,152,153] would seem to be concentrating on the evaluation of the electrical properties of such loaded phases in presence of pairing ion and their effects upon chromatography of ionic soluts.

Since testing of the above equations depends largely upon a knowledge of adsorbed pairing ion concentrations, it is intended to evaluate this model in the Discussion Section.

The different approaches outlined above concerning the process occurring during BRPIPC were dependent upon the possibility of ion pair formation in the mobile phase and/or adsorption of pairing ion by the C-18 surface [89,93,95,99,103,109]. Increasing evidence that such adsorption does occur has been taken as evidence supporting the contention that the process responsible for retention in such systems is essentially that of ion-exchange [98,99]. In addition, although many relationships between k' and adsorbed pairing ion concentration had been derived on a theoretical basis, these could not always be tested experimentally due to lack of experimental data concerning the adsorbed pairing ion concentration. Such data as was available [89.95,99,153], tended to be limited to relatively few pairing ions and to be restricted to fairly limited concentrations of such species in the mobile phase.

It seemed of fundamental importance to determine adsorption isotherms of several of the most widely used anionic and cationic pairing ions preparatory to attempting correlation of k' with mobile and stationary phase pairing ion concentrations. To standardise conditions, aqueous solution of buffer were to be used as mobile phase. The effect of organic modifier on adsorption was limited to one anionic and cationic pairing ion; the most highly adsorbed species being chosen in each case. ODS-Hypersil was standardised as the stationary phase, being representative of a modern capped octadecyl material.

It was intended to use pairing ions of widely differing hydrophobic character and to include types with and without surfactant properties since the suggestion had appeared in the literature that different mechanisms might occur depending on the nature of the pairing ion employed [93]. Also it was proposed to use sufficiently wide mobile phase concentration ranges of pairing ion to observe, not only the rise to the maximum capacity factor, but the subsequent reduction for which no satisfactory

explanation had been given. All measurements were made at ambient temperatures.

2.3.1 Apparatus

Column loading and column desorption were carried out using either an Applied Chromatography System (ACS 750/3) dual piston pump or a Pye-Unicam liquid chromatography (LCM2) pump. The pump used to drive the fluid to pack the column was Stansted fluid pump, Model (951). The detectors used were either a Pye-Unicam LC-3 or a modified Unicam SP500 single beam spectrometer. Recorders were either Servoscribe 1s (RE 541) or Vitatron. Columns for isotherm measurement were 250 x 5 mm stainless steel with Swagelok end fittings.

2.3.2 Materials

Stationary phase:- The stationary phase ODS-Hypersil was purchased from Shandon (London, Great Britain).

Cationic pairing ions:- Tetramethylammonium sulphate (TMA), tetraethylammonium bromide (TEA) and tetrabutylammonium sulphate (TBA) were obtained from Aldrich (Milwaukee, U.S.A.) in aqueous hydroxide form and neutralised with the appropriate acids to form the corresponding pairing-ion salts. Cetrimide (CETA) was obtained from I.C.I. Pharmaceuticals (Macclesfield, Great Britain).

Anionic pairing ions:- 1-Pentansulphonic acid (PANS) and 1-Octansulphonic acid (OCTS) as their sodium salt were obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) dl-Camphorsulphonic acid (CAMS) as its sodium salt was purchased from Koch-Light Labs. (Colnbrook, Great Britain). Sodium laurylsulphate (SLS) was obtained from BDH (Poole, Great Britain).

Solvent:- Acetonitrile was obtained from Rathburn (Walkerburn,

Great Britain). Water was double distilled and all other reagents were of AnalaR or comparable quality.

2.3.3 Column packing procedure

Columns were packed using the high pressure slurry apparatus shown schematically in Figure (1), and the method employed is similar to that recommended by Shandon Southern Products Ltd.[133].

About 4 g of packing material (ODS-Hypersil) was required for a 250 x 5 mm column. A slurry of ODS-Hypersil in isopropanol (10% w/v) was prepared and placed in ultrasonic bath for not less than 5 minutes. The slurry was then introduced into the slurry reservoir and the column was assembled. Hexane was used as the packing solvent and pressurised to 9000 p.s.i. The liquid valve was then opened to full position and the slurry pumped into the chromatographic column. After about 200 ml of hexane had been pumped through the column, the column and the slurry reservoir were inverted. The solvent reservoir was then filled with about 200 ml of methanol which was used to remove the hexane and the column was finally conditioned with about 100 ml of degassed 50% v/v methanol/water mixture. The liquid valve was then closed. When solvent stopped emerging from the column outlet, the gas supply was then turned off. The system was then left undisturbed for at least 15 minutes before the column was disconnected.

2.3.3.1 Adsorption isotherm measurements

The adsorption isotherms for all the pairing ions, both cationic and anionic, on ODS-Hypersil were determined by use of the desorption method [100]. In this method the adsorbent is allowed to equilibriate with the eluent containing a known concentration of pairing ion. The adsorbed material is then eluted by a desorbing agent. The mass of the pairing ion salt recovered is then determined by an appropriate analytical method.

In this study, the adsorption isotherms of TMA, TEA, TBA, CETA, PANS, CAMS, OCTS and SLS on ODS-Hypersil were determined by pumping several concentrations of a pairing ion through the column until the absorbance measured at suitable low wavelength was constant with time. Although the adsorbance was not linear with concentrations, the equilibrium state could be determined by this procedure.

Ethanol was used as the desorbing agent to remove adsorbed pairing ion, the mass of pairing ion salt deloaded was determined after evaporating the solvent under reduced pressure and was corrected for dead volume. The amount of pairing ion adsorbed by the column was calculated as follows:

$$Q_{ads} = W - (V_m \times C_m) \qquad -(29)$$

where Q_{ads} was the amount of pairing ion adsorbed by the column, W the weight of the salt deloaded, V_m was the dead volume of the column and Cm the mobile phase pairing ion concentration. The deloading process usually required one hundred column void volumes for strongly adsorbed ions such as CETA and SLS. Twice this volume was passed to ensure complete desorption.

2.3.3.2 Void volume determination

The maximum void volume of a column was determined as follows. The packed column was filled with water which was then recovered by passing about 90 ml of isopropanol through the column. The eluent was collected and diluted to 100 ml with isopropanol. The water was then measured by Karl Fischer titration and the void volume of the column calculated. This method is similar to that employed by other workers [99,136,137].

2.3.4.1 Results and discussion of the methods used in isotherms measurement

The desorption method was adopted after practically assessing two other common techniques employed in isotherm determination, these are the

break-through volume [122,142] and re-cycling methods [124].

In the break-through volume method, it is assumed that rate constants, K_{ads} and K_{des}, as represented by equation (30) are large, so that the time required to attain equilibrium is short.

$$(P^{\pm}\ C^{\mp})aq \underset{K_{des}}{\overset{K_{ads}}{\rightleftharpoons}} (P^{\pm}\ C^{\mp})org \qquad -(30)$$

The amount of pairing ion adsorbed onto the C-18 phase in a column can be calculated if the pairing ion column break-through time is known. Theoretically an idealised diagram as in Figure (2a) should be obtained on the recorder, when a fixed mobile phase pairing ion concentration is introduced onto the column, provided that the pairing ion involved can be detected. The amount of pairing ion adsorbed by the column can then be calculated as follows:

$$Q_{ads} = (V_r - V_m).C_m = (t_r - t_o).Fc.C_m \qquad -(31)$$

where V_r, V_m, t_r and t_o are defined in Section (2.1). Fc and C_m are the flow rate and the mobile phase pairing ion concentration respectively. All this information can be obtained from Figure (2a). In this study, however, such an idealised break-through volume diagram was seldom obtained. A more typical break-through volume diagram obtained experimentally is as shown in Figure (2b).

Under such circumstances, the value of t_r could not be determined accurately. Figure (2b) suggests that the equilibrium of the pairing ion between the mobile phase and the stationary phase is not at all rapid. This is not totally unexpected, as it can be envisaged that when the pairing ion is adsorbed onto the C-18 surface, the adsorbed pairing ion will exert an electrostatic repulsion on the pairing ion in the eluent. This effect will markedly slow down the rate of loading of the column.

The break-through volume method is applicable in determination of adsorption of a pairing ion only if the eluent pairing ion concentration is very low, usually below 2 mM [135]. For this reason following preliminary measurements, the break-through volume method was rejected in this study.

Another common method that has been used in isotherm determination is re-cycling of the eluent of known pairing concentration in a closed system until equilibrium is attained [124]. The concentration of the pairing ion in the mobile phase at equilibrium is then determined. The amount of pairing ion salt adsorbed by the column is then calculated using equation (32).

$$Q_{ads} = (C_s - C_m).V_t \qquad -(32)$$

C_s, C_m are the concentration of the pairing ion in the solvent before and after re-cycling and V_t is the total void volume of the closed system.

This method is time consuming and for the pairing ions used in the present study the determination of the mobile phase pairing ion concentration after re-cycling was difficult. In general direct measurement of the pairing ion concentration using UV spectrometric methods is inaccurate since most of the pairing ions employed in BRPIPC have very low molar absorptivities in the ultra-violet region. Some indirect methods [143,144] which make use of the formation of a complex with another strongly UV absorbing pairing ion or involve formation of coloured compounds with chemical reagents were not generally applicable to all pairing ions used. Such methods involve calibration, extraction, and dilution procedures if the pairing ion concentration is high. These factors tend to increase experimental error. The adsorption equilibrium of SLS of 2% w/v on ODS-Hypersil was evaluated by two methods, (a) re-cycling followed by photometric measurement of SLS-Methylene blue complex [143]

and (b) desorption followed by weighing SLS recovered.

The coefficient of variation measured on 4 replicates was 17.2% using re-cycling compared with 7.0% by the desorption method. SLS was chosen for the above comparison since the re-cycling method gave best results with this compound. Other pairing ions produced markedly larger errors using re-cycling. For the desorption method the most lightly loaded pairing ion even at the lowest concentration used produced a coefficient of variation of 16%.

The desorption method used in this investigation was checked for completeness of recovery by the following procedure. The column was allowed to equilibriate with a known concentration of mobile phase pairing ion. Ethanol was passed through the column and the eluent collected at the column outlet. Successive ten column volumes were collected into a small vial and ethanol was removed under reduced pressure. A plot of the cumulative weight of pairing ion eluted from the column against the number of column volumes was then produced. This plot for 2% w/v SLS in aqueous solution is shown in Figure (3). It is observed that the amount of SLS recovered reaches a plateau after about 100 column volumes.

The desorption method was used in all isotherm determination measurements.

Importance of column void volume in isotherm measurement

From equations (29), (31) and (32) it can be observed that in all methods of adsorption measurements, the calculation of quantity of pairing ion adsorbed by a column involves a column void volume correction. Inaccurate void volume measurement will inevitably lead to inaccurate adsorption measurements and consequently produce inaccurate isotherms.

This can be explained by inspecting equation (29),

$$Q_{ads} = W - V_m \cdot C_m \qquad -(29)$$

Let $$Q_{ads1} = W - V_{m1} \cdot C_m \qquad -(33)$$

and $$Q_{ads2} = W - V_{m2} \cdot C_m \qquad -(34)$$

where Q_{ads1} and Q_{ads2} are the quantities of a pairing ion adsorbed by a column calculated using different V_{m1} and V_{m2} respectively at a fixed concentration C_m and weight of pairing ion W deloaded.

Therefore $$Q_{ads2} - Q_{ads1} = C_m \cdot (V_{m1} - V_{m2}) \qquad -(35)$$

Let Q_{ads1} be the adsorption calculated using the accurate column void volume V_{m1}, the difference between Q_{ads1} lies in the difference between V_{m2} and V_{m1},

i.e. $$Q_{ads2} = Q_{ads1} + C_m \cdot (V_{m1} - V_{m2}) \qquad -(36)$$

If V_{m2} is smaller than V_{m1}, such calculation will produce an isotherm which does not reach a plateau. However, if an overestimated value of V_{m2} is used, such calculations produce an isotherm which exhibits a maximum.

Currently void volume determination of a column is usually made by measuring the retention volume of a so-called unretained solute [112,113]. Potassium bromide, sodium nitrate, potassium dichromate and potassium phosphate have all been used for this purpose [114]. It was found in this work that different void volumes were obtained depending on the particular unretained solute used. The void volumes estimated using several such solutes on a 250 x 5 mm ODS-Hypersil column are shown in Table (1), where they are compared with the maximum void volume resulting from Karl Fischer titration.

All the void volumes measured by using the so-called unretained solutes are smaller than the maximum void volume. This suggests that all the inorganic ions used are excluded by the ODS-Hypersil to some extent, while potassium dichromate is strongly excluded from the column.

In the present isotherm determination, using the desorption method, the maximum volume has been employed for the correction. This is thought justified as the pairing ion will occupy the maximum void volume in the column loading procedure and should be deducted from the amount of pairing ion that deloads from the column when the desorption process is completed. This will be of major importance for the weakly adsorbed pairing ions at high mobile phase concentrations.

2.3.4.2 Results and discussion of the adsorption isotherms

The adsorption isotherms obtained for the four cationic and four anionic pairing ions are shown in Figures (4a-b). When these are compared with the Giles classification [132] as shown in Figure (5), with the exception of the long chain alkyl surfactants CETA and SLS the isotherms are L type. These indicate a smooth rise to a plateau level characteristic of the particular pairing ion. At low concentration these isotherms would approximate to linearity or to the C type. On the other hand the two long chain alkyl surfactant pairing ions investigated, CETA and SLS, appear to be quite different and are markedly H type. This indicates appreciable adsorption even at very low aqueous concentrations. While many of the isotherms obtained could be fitted to the Langmuir type isotherm equation this would not be possible for CETA and SLS. In no case would the Freundlich equation provide an adequate fit over the whole concentration range studied since plateaus are observed in all cases. In any case, the purpose of the determination of an isotherm equation has been to allow evaluation of stationary phase pairing ion concentration from that in the mobile phase. Such evaluation is unneccessary if experimentally determined values of adsorbed concentrations are available. The appearance of various adsorption isotherms in the literature [89,98-103] serves only to verify that adsorption of pairing does occur. The resultant isotherm equations have

been used in attempts to relate k' with the mobile phase pairing ion concentration [89,99,124]. Such a procedure in this study would result in complex isotherms which would be unique to each pairing ion. The present results show that adsorption of all the tested pairing ions by the ODS-Hypersil does occur and that the extent of adsorption depends upon the nature of the pairing ions as well as the mobile phase concentrations. The different plateau levels for different pairing ions have been confirmed by recent work involving sodium octanesulphonate and SLS [99,103].

The adsorption of solvent on the surface of the C-18 support has been shown by several investigations [100,137-140]. It would appear then that adsorption of pairing ion occurs in competition with that of the solvent. The plateau loading capacity of the ODS-Hypersil appears from the present results to be related to the hydrophobicity of the particular pairing ion. Taking one criterion of the hydrophobicity of the pairing ion as that of the longest chain, since not all of the pairing ion can be desolvated, a reasonable correlation is obtained between the surface cationic pairing ion concentration at 0.05M aqueous concentration and the corresponding π hydrophobicity parameter [141],

$$Q_{ads}\ (\mu \text{ mol } g^{-1}) = 46.5\pi - 18 \qquad (R^2 = 0.991)$$

In the case of the anionic pairing ions, however, no such correlation is attempted due to the complex structure of CAMS which makes an estimate of its hydrophobicity difficult. A general increase in adsorption with increasing hydrophobicity is however apparent for the anionic ions studied.

It would appear that the primary force causing adsorption of pairing ion in such systems is hydrophobic in nature. It is likely that adsorption will be limited firstly by available surface area and also by the electrostatic layer which will accumulate on the C-18 surface with adsorption. Since the primary purpose of isotherm measurement in this study was to

verify that all pairing ions were adsorbed and to obtain equilibrium values for the adsorbed pairing ion concentration, no further discussion of the mechanism of adsorption is appropriate.

The unexpected adsorption isotherms for CETA and SLS which indicate high surface loadings even at very small mobile phase pairing ion concentrations is of considerable significance chromatographically. It may explain the previously reported findings that long equilibrium times are required when such very hydrophobic pairing ions are used at low concentrations [91,96]. It would also appear from these results that, in purely aqueous solution, regulation of the amount of adsorbed pairing ion is extremely critical and that high surface loadings will, in practice, always be obtained.

Adsorption isotherms in acetonitrile–water mixtures

Since the inclusion of organic modifier is general to regulate retention in chromatography, the effect of acetonitrile as organic modifier on the adsorption properties of pairing ions was thought to be of importance. This study was limited to the most highly adsorbed pairing ions used namely CETA and SLS. These compounds are also among the most commonly used pairing ions.

The results obtained are shown in Figures (6a) and (6b) respectively for three concentrations of acetronitrile, 0, 30 and 60% w/v. For both pairing ions, the isotherm is markedly altered by the addition of acetonitrile. Firstly the column loading is decreased; an effect which has been previously reported [99,108]. In addition the shape of the isotherm is seen to be drastically altered. The H-type isotherm exhibited in aqueous eluent are L-type in 30% v/v acetonitrile and approximate to linearity at 60% v/v the highest organic modifier concentration used. These results can be explained on the basis of decreased hydrophobicity of such pairing

ion in the more organic eluent. Alternatively it may be seen as the process of competive adsorption between hydrophobic pairing ion and acetonitrile for the C-18 surface. In either case as well as modifing the retention of solute during chromatography organic modifier is seen to alter the adsorption properties of the pairing ion.

Comparison with previous adsorption measurements on pairing ions

While direct comparison of the present results with those obtained by other workers is difficult due to arbitary choice of stationary and mobile phases, such a comparison is useful in establishing the validity of the present results.

The adsorption of OCTS on C-18 silica has been reported by several workers. Knox et al.[103] measured the adsorption at about 200 μmol g^{-1} on ODS-Hypersil from a mobile phase pairing ion concentration of 20 mM with a solvent composition of 0.02 M phosphate buffer-methanol 80:20, Deelder et al.[99] obtained 150 μmol g^{-1} from a solvent of phosphate buffer on Lichrosorb RP-18 at 5 mM eluent concentration. On the same stationary phase Johanson [135] recorded about 210 μmol g^{-1} from a solvent of phosphate buffer with 1.15% v/v 1-pentanol of eluent pairing ion concentration of 30 mM. In the present study ODS-Hypersil is shown to produce surface concentrations of the same pairing ion of 125, 180 and 200 μmol g^{-1} at eluent concentrations of 5, 20 and 30 mM respectively in pure water. This agreement is surprising in view of the experimental difficulties in isotherm measurement.

In the case of SLS the present results produce a maximum adsorption of 450 μmol g^{-1} from pure water which is comparable with the result of Deelder et al.[99] who obtained 500 μmol g^{-1} from solvent of phosphate buffer on Lichrosorb RP-18. The results obtained by Knox et al. on ODS-Hypersil from solvent of buffer-methanol 80:20 mixture were considerably higher at 760 μmol g^{-1} [238]. This may be attributed to errors in determination of column void volume.

The general effect established in the present study of adsorption of all commonly used pairing ion would appear to be substantiated by the findings of other workers on particular pairing ions [89,98-193].

2.4 Chromatography

It was intended to examine the relationships between solute chromatographic capacity factors and pairing ion concentration in BRPIPC. While several such investigations have appeared in the literature [93-105], they have dealt with limited numbers of pairing ions and, because of the large number of chromatographic variables the results obtained in different investigations are often not directly comparable. In this systematic study, it was intended to examine the chromatographic behaviour of solutes in the presence of all the pairing ions for which adsorption data had been obtained. It was anticipated that such a study could indicate if a constant mechanism of retention existed independent of the nature of the pairing ion. This was thought to be most relevant in the case of the cationic pairing ions where considerable differences exist between the symmetrical tertraalkyl compounds and the very asymmetric pairing ion used, CETA.

Various substituted benzoic acids were used as test solutes for the cationic pairing ions and a set of substituted aromatic amines were chosen for the anionic pairing ions. Sufficiently wide ranges of mobile phase pairing ion concentration were employed to achieve the reported decrease of capacity factor with high pairing ion concentration [92-103]. It has also been reported in the literature that the capacity factors of neutral solutes decrease in presence of surfactant pairing ion [123,131] and to examine this, 5-hydroxymethylfurfuraldehyde (5HMF) was included as a solute. This compound was chosen primarily because of its relevance in the D-glucose decomposition (Chapter 3) and also because it exhibited a suitable retention time in the purely aqueous solvents used.

From the results obtained, together with the experimentally obtained values of both mobile and stationary phase pairing ion concentrations it was intended to evaluate previously reported relationships between k' and pairing ion concentration. In particular it was intended to examine that proposed in Section (2.2.4) involving both ion pairing and ion exchange.

2.4.1 Apparatus

An Applied Chromatography Systems constant flow pump (750/3) with a Pye-Unicam (LC3) variable wavelength detector was used. Injection was via a Rheodyne 7125 valve with a 20 ul sample loop. Chromatographic colums were usually 50 x 5 mm, slurry packed as described in Section (2.3.3). For tetrabutylammonium sulphate a very short column 4 x 5 mm was made. Packing of this column was by direct addition of a thick ODS-Hypersil slurry in methanol to the column by spatula. All measurements were carried out at ambient temperature.

2.4.2 Materials

Solutes

2,4-Dimethylbenzoic acid (2,4-DMBA), naphthoic acid (NA) and 4-ethylbenzoic acid (4EBA) were obtained from Aldrich Chemicals (Milwaukee, WI, U.S.A). Benzoic acid (BA) and L-adrenaline-D-hydrogentartrate (AD) were obtained from Koch-Light Laboratories (Colnbrook, Great Britain). Aniline hydrochloride (AN) and benzylamine (BZ) were purchased from BDH (Poole, Great Britain). Dopamine hydrochloride (DOP) and 5-hydroxymethyl-furfuraldehyde (5HMF) were obtained from Sigma (St Louis, MO, U.S.A).

Buffer salt and solvent

Disodium hydrogen phosphate decahydrate was obtained from BDH (Poole, Great Britain) and solvents were as described in Section (2.3.2).

2.4.3 Procedure

The measurements were carried out in a standard buffer of 0.002 M disodium hydrogen phosphate decahydrate adjusted to pH 1.5 for the amines and pH 7.4 for the acids, by the addition of phosphoric acid. The appropriate concentration of pairing ion was then dissolved in this buffer. The column was equilibriated to this concentration before chromatographic results were recorded.

Flow rates of 1 to 3 cm^3 min^{-1} were employed and 254 nm was used as the detection wavelength for all solutes, except when CAMS was employed as pairing ion. In this case 235 nm was chosen as the wavelength of minimal background absorbance.

When TBA was used as pairing ion, rapid increases in back pressure were observed due to deterioration of the packing even at pH 7.4. This effect had previously been reported [92]. To overcome this, a precolumn of 40 x 5 mm dry packed with ODS-Hypersil was inserted before the injection valve. While this considerably lengthened the column life, frequent packing was necessary and the very short 4 x 5 mm column enabled such repacking to be accomplished rapidly. In addition the retention times for TBA were long and adequate k' values could be measured using such a short column length.

2.5.1 Results and discussion

The dotted curves in Figure (7 a-d) and (8 a-d) represent the variation of capacity factors (k') of the acidic and basic ionised solutes as a function of aqueous concentration of the cationic and anionic pairing ions respectively. Figures (9a) and (9b) and (10a) and (10b) are the results obtained for mixed eluents containing different proportions of organic modifier for pairing ions CETA and SLS respectively.

For all the pairing ions investigated, either in pure aqueous mobile phase or in presence of 30% and 60% v/v acetonitrile in the case of CETA

and SLS, clearly observed maxima are evident. The maxima are more pronounced the greater the hydrophobicity of both solute and pairing ion.

In the case of CETA and SLS in purely aqueous mobile phase, the only experimentally accessible k' values occur on the decreasing portion of the curve. This is interpreted as being a consequence of the H-type isotherm obtained for these compounds. That is, even at extremely low mobile phase concentrations an appreciable concentration of pairing ion is adsorbed on the stationary phase. The initial increase of k' with the aqueous mobile phase pairing ion concentration has been previously taken to support both the ion-pairing and ion-exchange interpretations of retention. Neither of these mechanisms, however, can account for the results of previous investigations, when high pairing ion concentration were used and a subsequent decrease in k' observed. Such a decrease in k' has been attributed to micelle formation in the case of soap e.g. CETA and SLS by previous workers [89,95,99]. In the present investigation, however, the decrease is observed to be general even for the non-surface active pairing ions.

The theoretical treatments of the ion-pairing mechanisms proposed by Schill [118] and Horvath et al. [93], which can be mathematically represented by equations (4) and (9) respectively;

$$k' = \phi . K_{eq} . [P^{\pm}]_{aq} \qquad -(4)$$

$$k' = \phi . \left(\frac{K_1 . K_3 . [P^{\pm}]_{aq} + K_2}{1 + K_1 . [P^{\pm}]_{aq}} \right) \qquad -(9)$$

both fail to describe the chromatographic behaviour observed generally in this study.

Also Equation (16) i.e.

$$k' = \phi . K_6 . \frac{[P^{\pm}\ C^{\mp}]_{org}}{[C^{\mp}]_{aq}} \qquad -(16)$$

which is derived based on the ion-exchange mechanism proposed by Knox et al.[89], indicates the following consequences.

If the C-18 surface is saturated with adsorbed pairing ion, $(P^{\pm}\ C^{\mp})org$, further increase in the mobile phase pairing ion concentration will only increase the value of the denominator, that is the counter ion concentration $[C^{\mp}]aq$ in the mobile phase. Thus the capacity factor of the ionic solute will decrease when the C-18 surface is saturated with $(P^{\pm}\ C^{\mp})org$. The results obtained in this investigation demonstrate that while the surface of the C-18 stationary phase is covered to different extents by different pairing ions, an essentially constant value of $[P^{\pm}\ C^{\mp}]org$ is indeed obtained. However, the decrease of the k' values occurs well before this constant value of $[P^{\pm}\ C^{\mp}]org$ is attained.

This suggests that a simple dynamic exchange mechanism is not appropriate to explain the chromatographic behaviour exhibited in BRPIPC. The combined approach outlined in Section (2.2.4) provides an alternative model for the prediction of the behaviour of k' and would seem to account for the decrease in k' observed.

Equation (28),

$$k' = \phi \cdot \left\{ \frac{K_6^{1/n} \cdot [P^{\pm}_n C^{n\mp}]^{1/n}org + K_2 \cdot [C^{n\mp}]^{1/n}aq}{K_1^{1/n} \cdot [P^{\pm}_n C^{n\mp}]aq + [C^{\mp}]^{1/n}aq} \right\} \qquad -(28)$$

derived in Section (2.2.4) predicts that k' will decrease as the term, $K_1^{1/n} \cdot [P^{\pm}_n C^{n\mp}]^{1/n}aq$, increases provided that this effect is not compensated for by a corresponding increase in, $K_6^{1/n} \cdot [P^{\pm}_n C^{n\mp}]^{1/n}org$. Thus, in principle, it is possible that, while the increase in k' is caused by increasing adsorption of pairing ion, the effect will be reduced and reversed by the formation of ion-pairs in the mobile phase as $[P^{\pm}_n C^{n\mp}]aq$ becomes large.

In order to test quantitatively the agreement of the equation (28) with the present experimental results, the following procedure was adopted.

For various aqueous concentrations of pairing ion $\left[P^{\pm}_{n}C^{n-}_{+}\right]_{aq}$ which can be obtained directly, the corresponding $\left[P^{\pm}_{n}C^{n-}_{+}\right]_{org}$ value was obtained from the appropriate isotherm. The value $\left[C^{n-}_{+}\right]_{aq}$ was calculated to include the concentration of the buffer. The constant K_2 in Equation (28) was estimated from the chromatographic result obtained in the absence of the pairing ion for each solute. The phase ratio, ϕ, for 5 μm ODS-Hypersil was taken as 142 m^2 cm^{-3} obtained from estimates of the total surface area of column packing and void volume of the column [155].

Using an iterative computer program (HOOKE1), (listed in the Appendix II), to obtain the best least mean squares fit of the experimental data k' vs $\left[P^{\pm}_{n}C^{n-}_{+}\right]$, the ion-exchange constant ($K_6^{1/n}$) and the ion-pairing constant, ($K_1^{1/n}$), in the mobile phase in Equation (28) were evaluated.

The results obtained for this model as represented by Equation (28) for various pairing ions and ionised solute species were unsatisfactory. The values of $K_6^{1/n}$ and $K_1^{1/n}$ of several solutes with the corresponding selected pairing ions are shown in Table (2). The agreement of calculated and experimental values is in general good as is demonstrated in the Appendix using NA as the solute and TEA as the pairing ion. Negative value of K_1, however, were obtained for several pairing ions. Also the stability constants for alkyltrimethylpyridinium alkylsulphate ion pairs range from 4×10^{-4} to 2×10^{-6} for alkyl groups between hexyl and hexadecyl [146]. The large value of 1.7 for K_1 obtained for 2,4-DMBA with TBA as pairing ion thus appears unrealistically high.

The physically unrealistic negative values or sometimes exceptionally high values of the ion-pairing constant K_1 obtained show the inadequacy of this model in BRPIPC.

In retrospect, there is one major shortcoming in all the previously discussed models. The phase ratio, ϕ, is in all cases regarded as

remaining constant on the addition of pairing ion to the eluent. It has been demonstrated in the above adsorption studies that substantial adsorption of pairing ion on the C-18 surface can occur so that part of the area of the organic phase would be permanently occupied by pairing ion. This will reduce the surface area available for solute adsorption. The assumption that ϕ is constant and independent of pairing ion concentration could thus be quite incorrect. In view of these inadequacies, another mechanism which is aimed at eliminating the above shortcomings has been derived.

2.5.2 Ion-exchange desolvation mechanism (IEDM)

In this mechanism it is attempted to accommodate the idea of a phase ratio variable with the adsorbed pairing ion concentration. It is assumed in this treatment that any formation of ion pairs in the mobile phase is negligible.

The IEDM may be represented as follows. The retention of an ionised solute in presence of pairing ion on the hydrophobic stationary phase may be considered as occurring by two separate processes. One is the desolvation of the solute on the C-18 surface. The extent of desolvation of the solute may be minimal in case of highly polar or ionised solutes resulting in small capacity factors. On the other hand the desolvation of a solute may be appreciable if the solute is hydrophobic even when ionised.

The nett retention volume is given by,

$$V_{r1} - V_m = K_{des} . A's$$

where V_{r1} is the measured retention volume, V_m the void volume in the column. A's the surface area of the stationary phase available for desolvation and K_{des} the desolvation constant of the ionised solute.

The other retention process suggested is an in situ ion-exchange equilibrium of the form

$$(P^{\pm}_{n}C^{n-}_{+})org + n(S^{-}_{+})aq \rightleftharpoons^{K_{IE}} n(P^{\pm}\ S^{-}_{+})org + (C^{n-}_{+})aq \qquad -(37)$$

An ion-exchange equilibrium constant can be written as

$$K_{IE} = \frac{\left[P^{\pm}\ S^{-}_{+}\right]^{n}org \ .\ \left[C^{-}_{+}\right]aq}{\left[P^{\pm}_{n}C^{n-}_{+}\right]org \ .\ \left[S^{-}_{+}\right]^{n}aq} \qquad -(38)$$

$$Ds^{-}_{+} = \frac{\left[P^{\pm}\ S^{-}_{+}\right]org}{\left[S^{-}_{+}\right]aq} \qquad -(39)$$

by substitution for $\left[P^{\pm}\ S^{-}_{+}\right]org$ we have

$$Ds^{-}_{+} = \left(\frac{K_{IE} \ .\ \left[P^{\pm}_{n}\ C^{n-}_{+}\right]org}{\left[C^{n-}_{+}\right]aq}\right)^{1/n} \qquad -(40)$$

Such a relationship alone does not allow for any desolvation of the ion-pair on the surface of the C-18 stationary phase due to the hydrophobicity of the solute. It does not account for the differences in distribution and thus retention among different solutes other than in purely electrostatic terms. In the light of this it is argued that desolvation of the ionised solute species on the C-18 surface occurs after the neutralisation of the charge by the ion-exchange interaction. The tendency for desolvation will be proportional to the hydrophobicity of the neutralised solute as described by a desolvation constant K'_{des}, which will act to increase the electrostatic effect of the ion-exchange process. K'_{des} although analogous to K_{des} may be of different magnitude since it refers to a solute with no nett ionic charge.

The retention of the solute in the ion-exchange-desolvation process will be proportional to the area A's of the C-18 surface available for desolvation. Thus the nett retention for an ionised solute retained by ion-exchange-desolvation is given by

$$V_{r2} - V_m = K'_{des} \cdot A's \cdot \left(\frac{K_{IE} \cdot \left[P^{\pm}_{n} C^{n-}_{+} \right] org}{C^{n-}_{+} aq} \right)^{1/n} \qquad -(41)$$

The total nett retention volume $(V_{rT} - V_m)$ is given by addition of Equations (29) and (44) as

$$V_{rT} - V_m = K'_{des} \cdot A's + K'_{des} \cdot A's \cdot \left(\frac{K_{IE} \cdot \left[P^{\pm}_{n} C^{n-}_{+} \right] org}{\left[C^{n-}_{+} \right] aq} \right)^{1/n} \qquad -(42)$$

and the solute capacity k' by

$$k' = \frac{1}{V_m} \cdot \left\{ K_{des} \cdot A's + K'_{des} \cdot A's \cdot \left(\frac{K_{IE} \cdot \left[P^{\pm}_{n} C^{n-}_{+} \right] org}{\left[C^{n-}_{+} \right] aq} \right)^{1/n} \right\}$$

$$= \frac{A's}{V_m} \cdot \left\{ K_{des} + K'_{des} \cdot \left(\frac{K_{IE} \cdot \left[P^{\pm}_{n} C^{n-}_{+} \right] org}{\left[C^{n-}_{+} \right] aq} \right)^{1/n} \right\} \qquad -(43)$$

A's is related to the total surface area of the C-18 stationary phase, As, by the relationship

$$A's = As - \left[P^{\pm}_{n} C^{n-}_{+} \right] org \cdot Ap \qquad -(44)$$

where Ap is the area per mole occupied by a particular absorbed pairing ion. As, is difficult to define but can be thought of as the effective area available for adsorption of any species in terms of C-18 surface.

Substituting A's in the expression for k', Equation (45) is obtained

$$k' = \frac{1}{V_m} \cdot \left(As - \left[P^{\pm}_{n} C^{n-}_{+} \right] org \cdot Ap \right) \cdot \left\{ K_{des} + K'_{des} \cdot \left(\frac{K_{IE} \cdot \left[P^{\pm}_{n} C^{n-}_{+} \right] org}{\left[C^{n-}_{+} \right] aq} \right)^{1/n} \right\} \qquad -(45)$$

Equation (45) is similar to (28), except that the phase ratio ϕ in Equation (45) is dependent on the adsorbed pairing ion concentration. Also the ion-pair constant $K_1^{1/n}$ of Equation (28) is regarded as zero in Equation (45) and the ion-exchange constant $K_6^{1/n}$ is modified into a composite constant $K'_{des} \cdot K_{IE}^{1/n}$.

Equation (45) relates the k' of a given solute with the adsorbed

pairing ion concentration and mobile phase counter ion concentration, and also takes into account the effective area C-18 available for desolvation. It shows a much more complex dependence of k' on adsorbed pairing ion concentration than has hitherto been suggested. The IEDM can be diagramatically represented by Figure (11).

The form of equation (45) can be seen more readily if the situation of monovalent counter ion only is considered. Equation (45) can be rearranged after settingnto unity as

$$k' = \frac{1}{V_m} \cdot \left\{ As \cdot K_{des} - K_{des} \cdot [P^{\pm} C^{\mp}]_{org} \cdot Ap + K'_{des} \cdot \frac{K_{IE} \cdot [P^{\pm} C^{\mp}]_{org}}{[C^{\mp}]_{aq}} - K'_{des} \cdot K_{IE} \cdot Ap \cdot \frac{[P^{\pm} C^{\mp}]^2_{org}}{[C^{\mp}]_{aq}} \right\} - (4$$

The k' is thus expressed as a quadratic function of $[P^{\pm} C^{\mp}]_{org}$ which exhibits a maximum value. This is in agreement with the results obtained by numerous workers when considering the k' dependence on aqueous pairing concentration. The decrease in k' of the ionic solute as demonstrated in Figures (7-10) may be due to two quite dissimilar effects as interpreted by this model. The C-18 surface area available for desolvation subsequent to ion-exchange will decrease according to the term $(As - [P^{\pm}_n C^{n\mp}]_{org} \cdot Ap)$ when the loading of the pairing on the C-18 surface increases. Also, k' will decrease as $[C^{n\mp}]_{aq}$ increases as a result of added pairing ion salt. Under the experimental conditions chosen, using low ionic strength buffer to minimise purely hydrophobic chromatography [72], it is not possible to separate these two effect i.e. increase in $[P^{\pm}_n C^{\mp}]_{aq}$ will lead to increase in $[P^{\pm}_n C^{n\mp}]_{org}$, according to the isotherm, thus decreasing the available surface area and will also increase the $[C^{n\mp}]_{aq}$ directly.

Equation (45) was subjected to a similar quantitative assessment to that carried out for the previous model. The program used, (HOOKE2), again provided a comparison between calculated and experimental values of

k' and provided values for the constant $K'_{des} \cdot K_{IE}$. (HOOKE2) is listed in the Appendix II.

The following procedure was adopted to provide the required data. The values As, $\left[P^{\pm}_{n} C^{n-}_{+}\right]$ org and $\left[C^{n-}_{+}\right]$ aq at a given aqueous pairing ion concentration were obtained as discussed previously. K_{des} was estimated from the chromatographic results in absence of pairing ion for each solute. Ap was calculated on the basis of accepted atomic dimensions, bond angles and lengths for each of the pairing ions in the fully extended conformation using data and calculations contained in the CAMSEQ [147] software system. The areas of symmetrical pairing ions were calculated as circles with alkyl chain length as radius and that of CETA, PANS, OCTS, SLS and CAMS as molecules in a flat position on the adsorbed surface.

The resulting graphs of calculated capacity factors against aqueous pairing ion concentration from Equation (45) are shown in Figures (7–10) as full lines.

To further demonstrate the fit of the proposed IEDM, Figures (12a–d) and (13a–d), (14a–b) and (15a–b) show experimental (dotted lines) and calculated (full lines) values of k' as a function of adsorbed pairing ion concentration $\left[P^{\pm}_{n} C^{n-}_{+}\right]$ org. The experimental curves in these plots indicate more forcefully that k' will decrease after the maxima even though the $\left[P^{\pm}_{n} C^{n-}_{+}\right]$ org values continue to increase. When $\left[P^{\pm}_{n} C^{n-}_{+}\right]$ org is essentially constant, the decrease of k' becomes more drastic on further increase in mobile phase pairing ion concentration. The decreasing section of the curve is accounted for by the sum of two effects, namely decreased in C–18 surface available for desolvation and increasing eluent counter ion concentration as a result of added pairing ion salt. This latter effect is most obviously demonstrated in the cases of CETA and SLS in water mobile phase. While the adsorbed pairing ion concentration is constant as indicated by the plateau region of the isotherm, k' decreases markedly on increasing mobile phase pairing ion concentration.

The effect of counter ion in the proposed IEDM is similar to that in the original ion-exchange process. Included in the IEDM ideas, however, is the possibility that pairing ion salt which can not be adsorbed by the surface will function as additional counter ion.

In a recent publication , Sorel et al.[238] have criticised this explanation in the case of CETA. They suggest that micelle formation occurs at the same mobile phase pairing ion concentration and that this will contribute to the very marked decrease in k'. While such an alternative explanation is possible for CETA it would not be expected to apply in the case of the other cationic pairing ions studied.

Figures (12-15) showing that the magnitudes of the experimental and calculates k' values are in good agreement over such a wide range of concentration of $\left[P^{\pm}_{n}C^{n-}_{+}\right]_{org}$ based on the best value of an adjustable constant is taken as evidence that the proposed IEDM is adequate to explain the retention of the ionised solutes in BRPIPC.

The optimum values of the composite constant $K'_{des}.K_{IE}^{1/n}$ obtained for cationic and anionic pairing ions in pure water mobile phase are shown in Tables (3a) and (3b), together with the average values K_{des} obtained for a particular solute in absence of pairing ion. The $K_{des}.K_{IE}^{1/n}$ values represent the equilibrium constants of the ion-exchange-desolvation process and are seen to increase with the hydrophobicity of a solute for all pairing ion as does K_{des}. Considerable fluctuation however is apparent for each solute as a function of pairing ion.

In order to separate the components of desolvation and ion-exchange, the approximation is made that the ratio between K'_{des} and K_{des} is constant for a given pairing ion which allows the relative values of ion-exchange constants K'_{IE}, to be estimated. This assumption appears to be justified in the light of recent studies of the retention ratio of carboxylic acids as a function of pH where k' of the acids have been

measured in both non-ionised and fully ionised states [73,124].

The relative values of K'_{IE} involving the above assumption are shown in Tables (4a) and (4b). In the case of CETA pairing ion in water, the experimental data points for the more hydrophobic solutes NA and 4EBA were not sufficiently representative of the parabolic shape of the curve to enable values of $K'_{des}.K_{IE}^{1/n}$ to be evaluated accurately.

It can be seen from Tables (4a) and (4b) that the K'_{IE} values are substantially constant for all solutes within a given pairing ion and even for different pairing ions of both charges. This is taken to indicate that the electrostatic binding thought to be operative is the same for all pairing ions and solutes. The term $K'_{des}.K_{IE}^{1/n}$ is the major factor dictating retention and resolution of the solutes, since in all cases, this term is substantially larger than K_{des}.

In the cases of eluents incorporating acetonitrile, the values of k' in absence of pairing ion are very small and for the higher concentration of acetonitrile apparently negative [148]. For the mixed solvent measurements, the $K'_{des}.K_{IE}^{1/n}$ values are shown as a function of acetonitrile concentration in Table (5). As would be expected, their values are lower than in pure aqueous solvent due to competitive adsorption between acetonitrile and pairing ion on the C-18 surface. Such an effect will reduce the value of K'_{des} and also reduce the loading of the pairing ion on the C-18 surface.

The apparent success of the IEDM in the interpretation of the chromatographic data of these ionic solutes leads to the conclusion that the failure of the combined ion-pairing ion-exchange mechanism is due only to the lack of consideration of the variation in phase ratio. To test this, the variable phase ratio employed in Equation (45) was incorporated in Equation (28) resulting in Equation (47) shown below.

$$k' = \frac{(As - Ap.\left[P^{\pm}_{n}C^{n-}_{+}\right]org)}{V_m} \cdot \left\{ \frac{K_6^{1/n}.\left[P^{\pm}_{n}C^{n-}_{+}\right]^{1/n}org + K_2.\left[C^{n-}_{+}\right]^{1/n}aq}{K_1^{1/n}.\left[P^{\pm}_{n}C^{n-}_{+}\right]^{1/n}aq + \left[C^{n-}_{+}\right]^{1/n}aq} \right\} \quad -(47)$$

Equation (47) was again subjected for curve fitting analysis to test its fit with the experimental results. The results obtained, as shown in Appendix II, indeed show minor improvement of the fit. The values of the ion-pair constant $K_1^{1/n}$ remain negative. This further suggests that the inclusion of an ion-pairing equilibrium in BRPIPC may not be appropriate.

2.5.3 Effect of adsorbed pairing ions concentration on the capacity factor of a non-ionic solute in BRPIPC

So far few workers have reported the effect of mobile phase ion concentration on the retention ratio of non-ionic solutes [96,108,123,131]. According to the IEDM, as mathematically represented in Equation (45), increase in adsorbed pairing ion concentration will decrease the phase ratio. This will have the effect of reducing the retention of non-ionic solutes.

In the absence of ion-exchange, Equation (45) is reduced to

$$k' = \frac{K_{des}}{V_m} \cdot (As - [P^{\pm}_n C^{n-}_{+}]\,org \cdot Ap) \qquad -(48)$$

Equation (48) clearly indicates that the increase in the area occupied by the pairing ion will reduce the k' of an non-ionic solute. This is consistent with the experimental results of other workers [96,123,131] and in some investigations where pairing ion appeared to have no effect on non-ionic solutes the alteration in phase ratio would be expected to be minimal. Theoretically Equation (48) predicts that the decrease in k' should have a linear dependence on $[P^{\pm}_n C^{n-}_{+}]\,org.Ap$. The study of the chromatographic behaviour of a neutral compound as a function of the adsorbed pairing ion concentration will yield further evidence of the surface coverage aspect of the proposed IEDM.

In this investigation 5HMF was chosen as the solute. The plot of

k' against $\left[P^{+}_{-n}C^{n-}_{+}\right]_{org.Ap}$ of all pairing ion for 5HMF is shown in Figure (16). For all pairing ions at all concentrations, there is a marked reduction of 5HMF capacity factor, which tends to a small constant value. The theoretical line of Equation (48) appears to underestimate the surface exclusion effect of the adsorbed pairing ions of a polar nutral species. This may suggest that the effective reduction in C-18 surface area by the adsorbed pairing ion is greater than the actual surface occupied.

This can be explained as in Figure (17), where the area available for desolvation of 5HMF is (As - eA) instead of (As - aA), where (eA) and (aA) are the total effective and actual areas respectively occupied by the adsorbed pairing ion. Since estimation of (eA) is difficult if not impossible, because it depends on the shape and the arrangement of the pairing ions on the C-18 surface, Equations (45) and (48) can not be further modified to fit the experimental results. However, it is possible that the surface area aspect of the present model is quantitatively inadequate due to our incomplete understanding of the physical nature of the C-18 surface in the presence and absence of organic modifier and pairing ion [69,70,149,150].

Equation (48) has also ignored the influence on the magnitude of K_{des} of the presence of pairing ion in the mobile phase. According to the solvophobic theory [71], variation of surface tension and ionic strength of the mobile phase will affect the capacity factor of a solute. Consequently this implies that the desolvation constant K_{des} will be affected by the added pairing ion salt which will, in effect, increase the ionic strength, but may also decrease the surface tension of the solvent especially if the pairing ion is surface active. That means K_{des} may not be constant but may depend upon mobile phase pairing ion concentration. The inclusion of the ionic strength and surface tension of the solvent in

Equation (48) in order to allow for the variation of K_{des} by the added pairing ion has not been attempted. A recent study by Graham et al.[131], however, suggests that the effect of the surface tension and ionic strength of the solvent in retention of a non-ionic solute in BRPIPC is minimal.

These limitations do not invalidate the generality of the proposed model and any alternation in the As, Ap and K_{des} values used would only have the effect of altering the absolute magnitude of the derived constants. The exclusion of 5HMF from the C-18 surface in presence of adsorbed pairing ion further casts doubt upon the importance of ion-pair formation in BRPIPC. Any ion-pair complex formed will be non-ionic but fairly polar, once formed in the mobile phase. It is likely therefore that it will behave as does 5HMF and tend to be excluded from the C-18 surface.

2.6 Conclusions

The present investigation indicates that the loading of C-18 phase with pairing ions of appropriate charges in aqueous eluents is substantial. This is especially true in the case of long chain pairing ions having surfactant properties. Also these show considerable adsorption at very low pairing ion concentrations and may produce unacceptably high k' values when used with solutes which are retained to some extent in the absence of pairing ion unless appreciable concentrations of organic modifier are used to alter the H-type isotherm behaviour. Less strongly adsorbed pairing ions provide a more sensitive method of adjusting retention time by pairing ion concentration. The ion-exchange-desolvation model appears adequate to represent the behaviour of both anionic and cationic solutes in presence of many hydrophobic pairing ions. The magnitude of the constants relating to the ion-exchange aspect of the proposed mechanism indicate that the electrostatic binding is approximately constant for all

solutes and pairing ions. This model also predicts the chromatographic behaviour satisfactorily in mixed eluents containing both water and organic modifier. Theoretically, the capacity factor of an ionic solute can be adjusted to any required magnitude by use of suitable pairing ions and mobile phase systems. In practice the k' value of a solute is usually adjusted to between 5 and 20 and the selection of the pairing ion becomes critical. According to the IEDM, the choice of the pairing ion used depends on its isotherm on the C-18 surface in the mobile phase system selected. If possible, the concentration of the pairing ion used should not be at the proportional region of the isotherm. This is because at the proportional region, slight variation of the mobile phase pairing ion concentration will markedly alter its loading on the C-18 surface resulting in drastic alterations in the k' of the solutes. Ideally, to obtain most reproducible retention times, the concentration of the pairing ion used in BRPIPC analysis should be at or near the plateau region of the isotherm. Under such circumstances, the adsorbed pairing ion concentration is very insensitive to the mobile phase concentration. Slight variation in mobile phase pairing ion concentration will only alter the solvent counter ion concentration and will usually produce minimal effect [123]. Unfortunately such situations can seldom be attained in practice. The optimal system used for analysis of a given solute can not be predicted theoretically unless the composite constant $K'_{des} \cdot K_{IE}^{1/n}$ of the pairing ion and its adsorption isotherm on the organic support are known.

However, the adsorption isotherms of the several commonly used anionic and cationic pairing ions on the ODS-Hypersil measured will at least give some help in the initial selection of a pairing ion for the chromatographic analysis of ionic solutes using BRPIPC. For example it will be inappropriate to use CETA as the pairing ion to retain an acid in pure water mobile phase, as the k' will be too large, whilst the use of

TMA as the pairing ion in 60% v/v solute will result in minimal retention due to the very low C-18 loading of TMA at such solvent compositions.

It has also been demonstrated in this study that, while different pairing ions of the same charge may have different adsorption properties on the organic support, there is no difference in the mechanism involved in the retention of the solutes. This suggests that although the use of different pairing ions may affect the capacity factors of the ionic solutes due to their different loading capacities, it will have little effect in changing the selectivities among compounds. Selectivity is a thermodynamic effect [133] which can be represented by the desolvation constant K'_{des} in Equation (45) of an ionic solute after its charge has been neutralised by the adsorbed pairing ion. The magnitude of K'_{des} in turn depends on the solvent employed in a chromatographic system. Thus if two cationic solutes can not be separated by use of the pairing ion OCTS, it is unlikely that separation will be achieved by changing to SLS as the pairing ion, provided the k' values in both systems are comparable.

Recent work appearing in the literature tends to contradict this prediction. It has been shown in the case of the separation of adrenaline and noradrenaline using BRPIPC that different pairing ions can alter selectivity among solutes [153]. This is currently being explained in terms of electrical double layer ideas involving binding of counter ion and represents an advance on the present work which does not attempt to quantify electrostatic effects. Later measurements in this study verify the alteration of selectivity by different pairing ions and this will be outlined in Chapter (3).

As with all chromatography, other than the gel-permation mode, in BRPIPC the major factor controlling retention and separation of solutes remains the solvent used in the chromatographic system.

The behaviour of 5HMF, a fairly polar neutral solute, shows a marked

decrease in retention with increasing pairing ion concentration for all pairing ions studied. This casts doubt upon the importance of ion-pair formation in the mobile phase as a process likely to contribute to the retention of ionic solute species in BRPIPC. The difficulty of quantifying the phase ratio in presence of pairing ion may be a factor resulting in the inadequacy of this model in predicting quantitatively the behaviour of the neutral test substance 5HMF.

2.7 Supplement - Mathematical treatment of the IEDM involving the use of multivalent pairing ion and counter ion in the chromatography of multivalent solutes

The IEDM proposed can be extended to the study of chromatographic behaviour involving the use of multivalent pairing and counter ions in the retention of a multivalent solute in BRPIPC. The derivation is similar to that described previously with minor modifications.

$$m(P^{l+}_{-n}C^{n-}_{+l}) + ln(S^{m-}_{+})aq \rightleftharpoons^{K_{IE}} n(P^{l+}_{-m}S^{m-}_{+l})org + lm(C^{n-}_{+})aq \qquad -(49)$$

l,m and n are the valencies of the pairing ion, solute and counter ion respectively.

$$\text{Therefore } K_{IE} = \frac{\left[P^{l+}_{-m}S^{m-}_{+l}\right]^{n}org \cdot \left[C^{n-}_{+}\right]^{lm}aq}{\left[P^{l+}_{-n}C^{n-}_{+l}\right]^{m}org \cdot \left[S^{m-}_{+}\right]^{ln}aq} \qquad -(50)$$

$$l(S^{m-}_{+})aq \rightleftharpoons^{Ds^{-}_{+}} (P^{l+}_{-m}S^{m-}_{+l})org \qquad -(51)$$

$$Ds^{-}_{+} = \frac{\left[P^{l+}_{-m}S^{m-}_{+l}\right]org}{\left[S^{m-}_{+}\right]^{l}aq} \qquad -(52)$$

$$= \frac{K_{IE}^{1/n} \cdot \left[P^{l+}_{-n}C^{n+}_{-l}\right]^{m/n}org}{\left[C^{n-}_{+}\right]^{lm/n}aq}$$

$$= \left(\frac{K_{IE} \cdot \left[P^{l+}_{-n}C^{n-}_{+l}\right]^{m}org}{\left[C^{n-}_{+}\right]^{lm}}\right)^{1/n} \qquad -(53)$$

Substituting Equation (53) into (45), the k' of a multivalent solute (S^{m-}_{+}) can be expressed as a function of the adsorbed pairing ion and the mobile phase counter ion concentrations, and Equation (54) is obtained.

$$k' = \frac{1}{V_m} \cdot (As - \left[P^{l+}_{-n} C^{n-}_{+1}\right]org.Ap) \cdot \left\{ K_{des} + K'_{des} \cdot \left[K_{IE}^{1/n} \cdot \left(\frac{\left[P^{l+}_{-n} C^{m-}_{+1}\right]org}{\left[C^{n-}_{+}\right]^{l}aq} \right)^{m/n} \right] \right\} \quad -(54)$$

Equation (54) indicates that the k' of a multivalent solute will be extremely sensitive to both adsorbed pairing ion ($P^{l+}_{-n} C^{n-}_{+1}$)org and aqueous counter ion (C^{n-}_{+})aq concentrations. Such phenomena have been reported by Karger et al. [94].

However, Equation (54) has taken a very simplistic assumption namely that for example, a divalent solute will react with two monovalent adsorbed pairing ions or that a trivalent adsorbed pairing ion will bind to three monovalent solutes. In practice such a stoichiometric fitting may not occur and partial neutralisation of charges may take place. That is a divalent solute may only react with only one monovalent pairing ion or a trivalent pairing ion may only bind to one or two monovalent solutes. If partial neutralisation does occur, it will give rise to a series of ion-exchange constants of the pairing ion and different desolvation constants for the partially and fully neutralised solutes. This will inevitably cause the mathematical treatment of the IEDM to be extremely complex and difficult to apply in practice to predict the retention behavour of the solute in such situations.

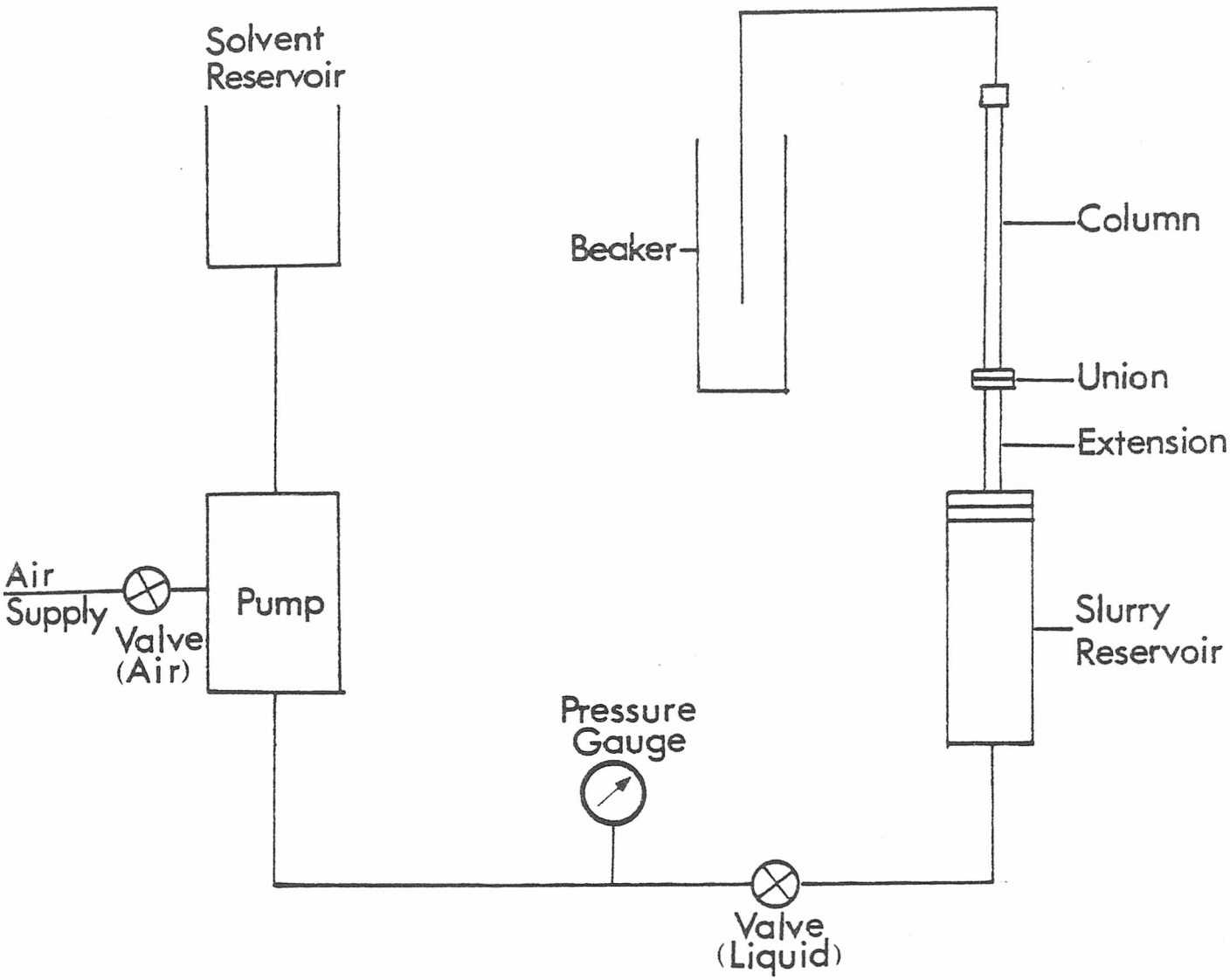


Figure 1 Schematic of high pressure slurry packing apparatus.

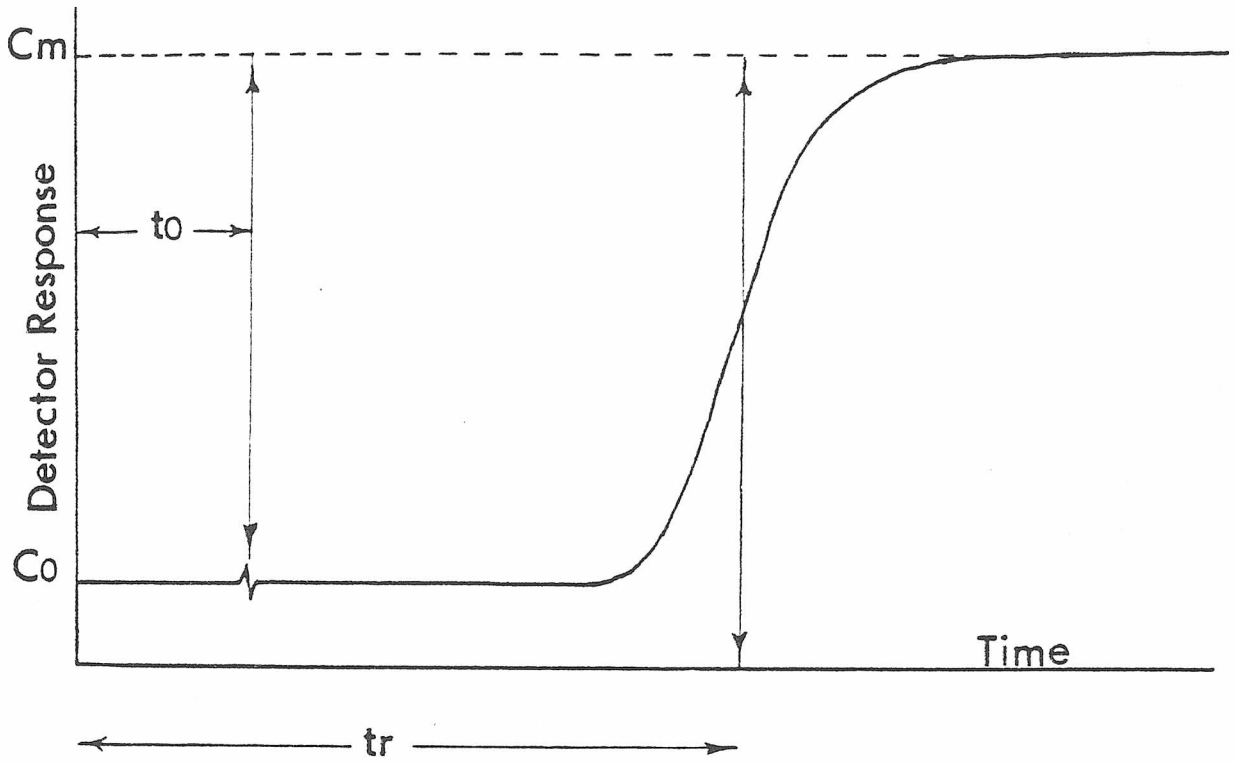


Figure 2a An idealized column break through volume diagram.

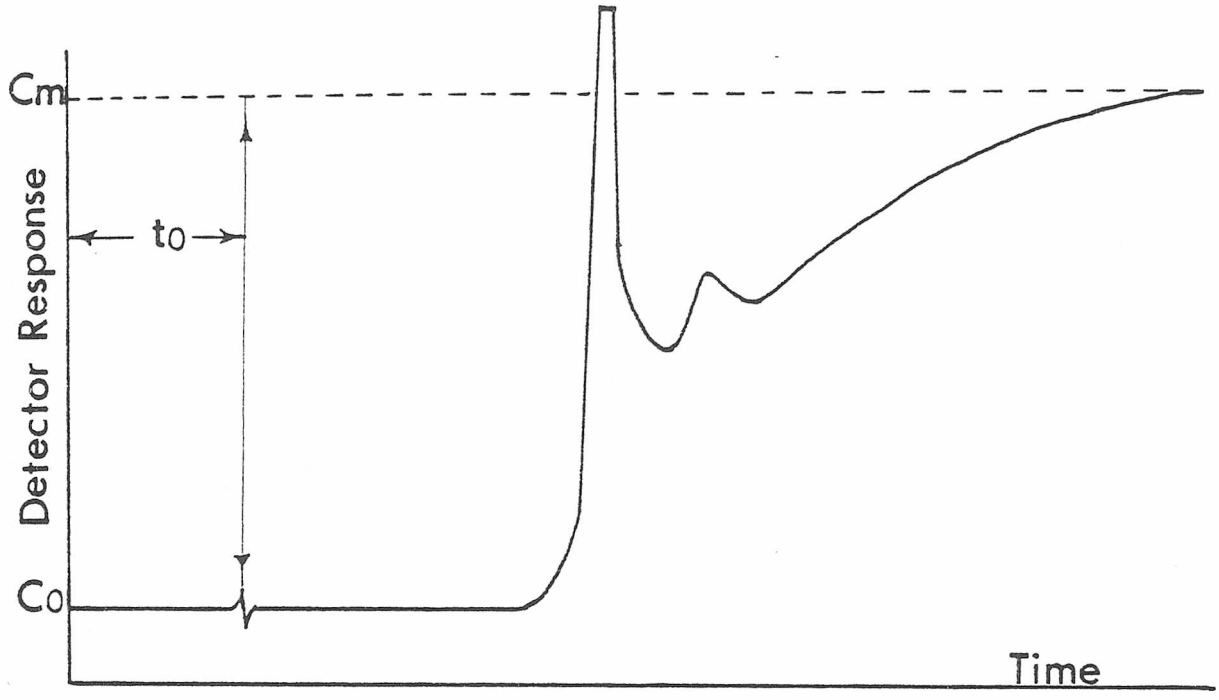


Figure 2b A typical column break through volume diagram obtained experimentally.

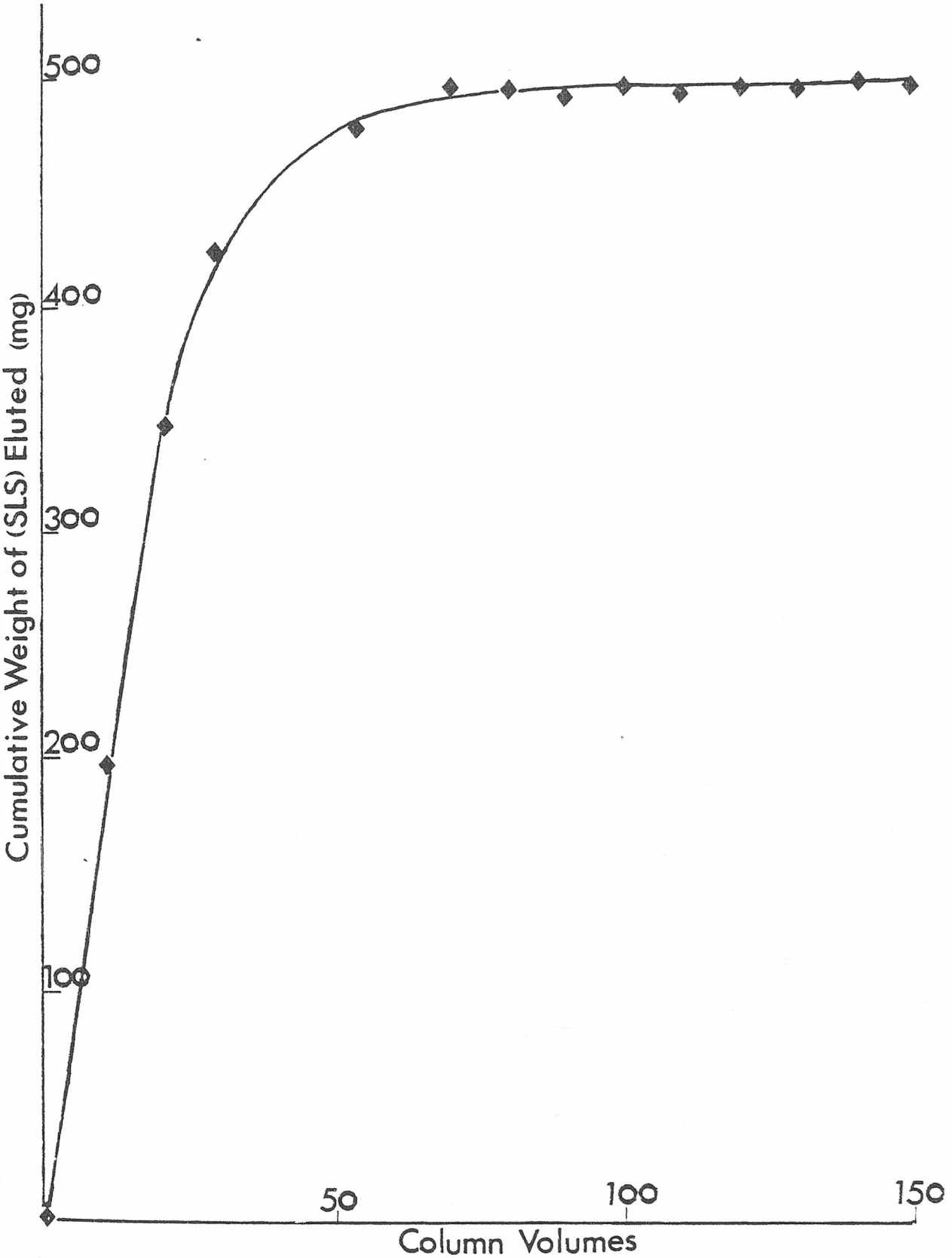


Figure 3 Desorption of (SLS) from a loaded column (250 x 5 mm), mobile phase concentration 2% w/v, using ethanol.

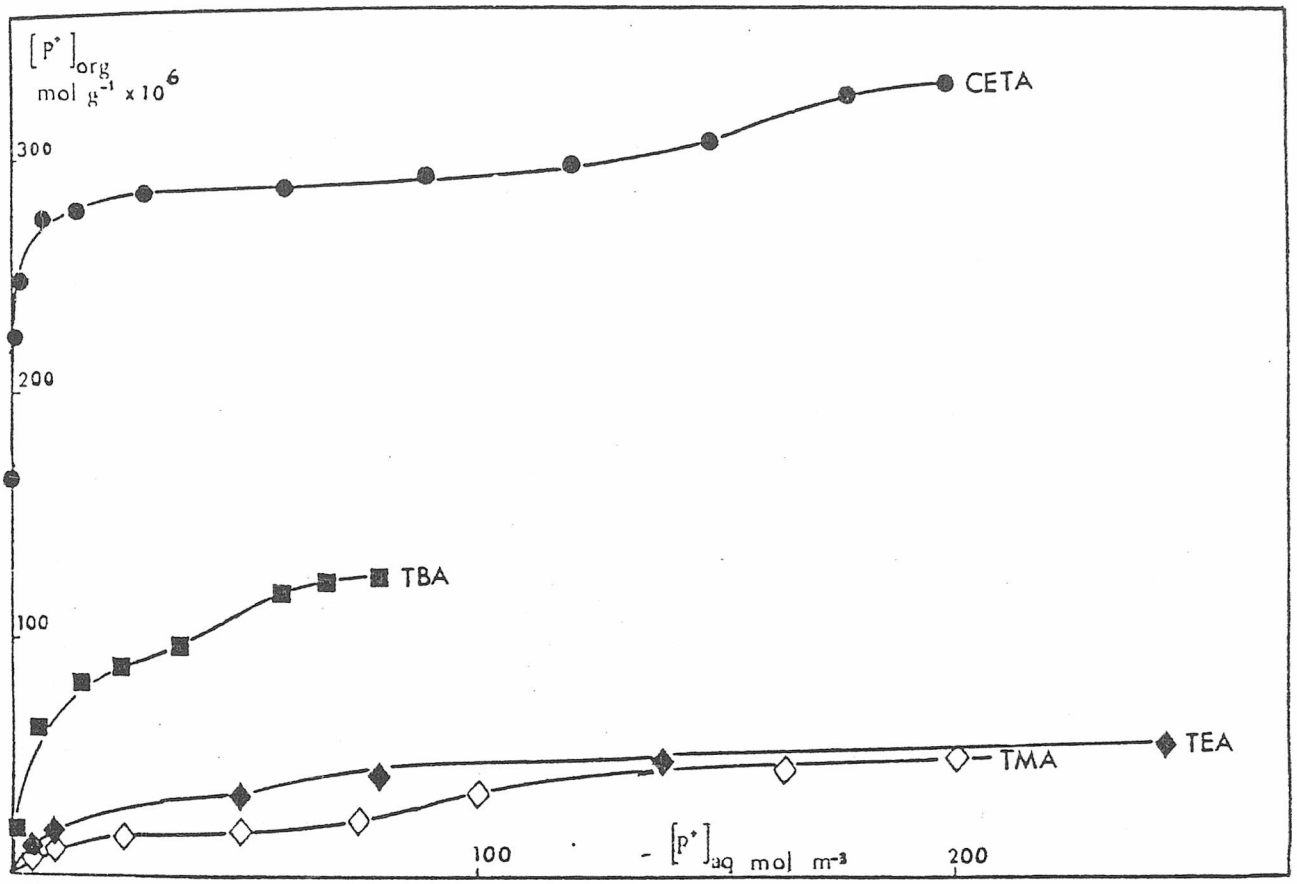


Figure 4a Adsorption isotherms for various cationic pairing ions on ODS-Hypersil from water.

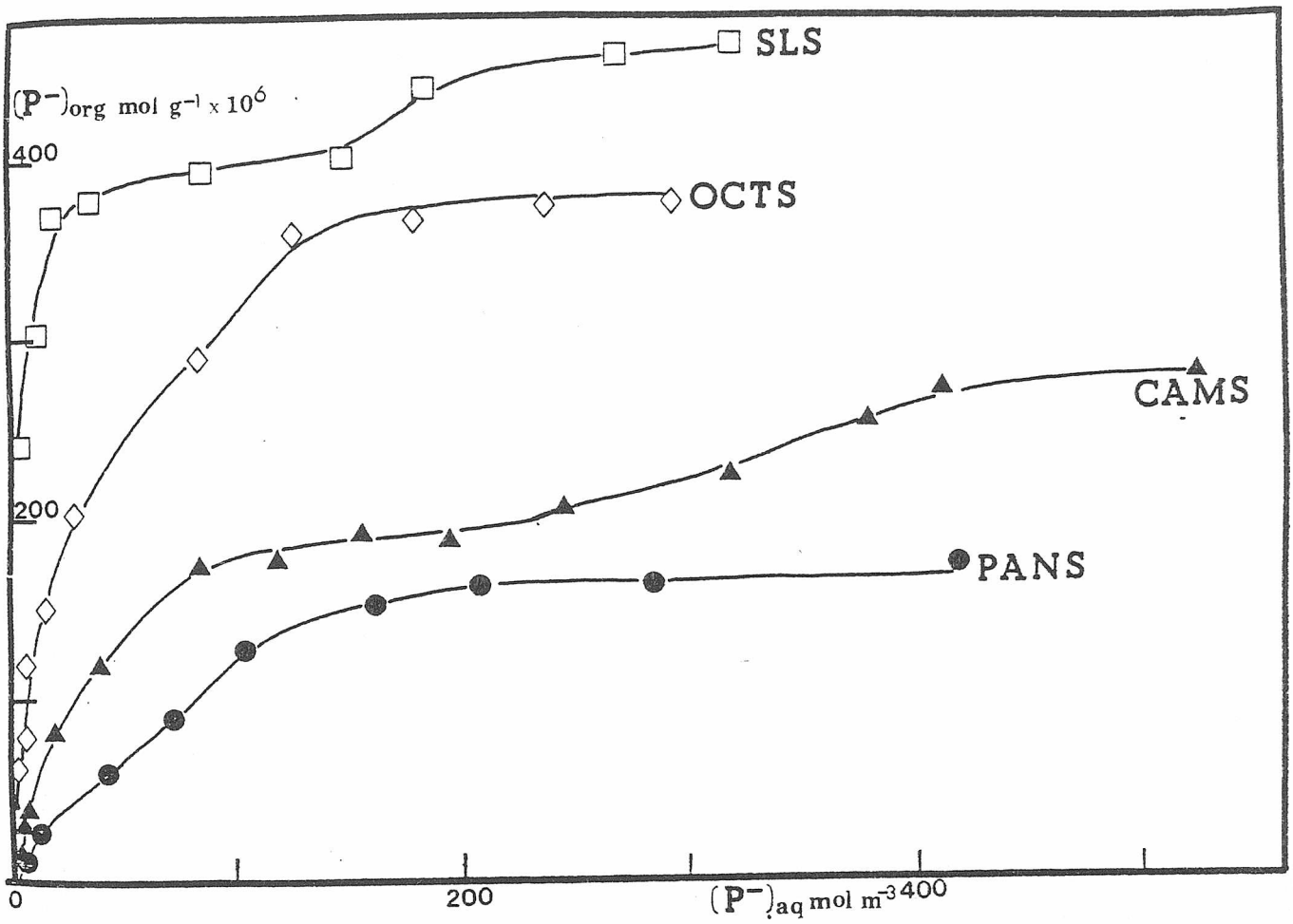


Figure 4b Adsorption isotherms for various anionic pairing ions on ODS-Hypersil from water.

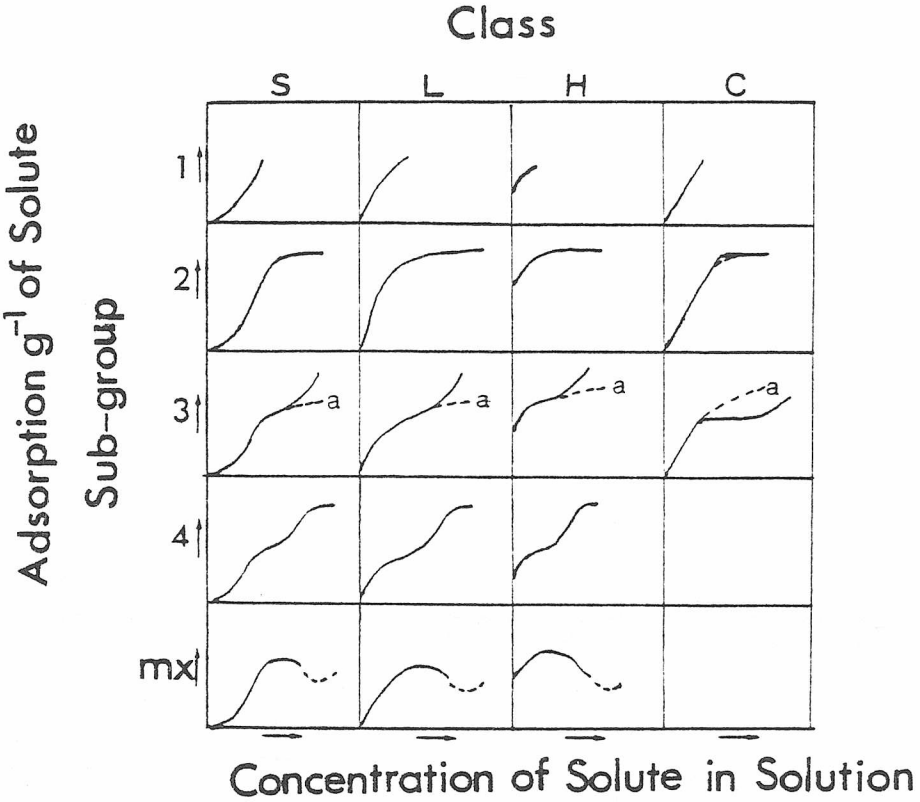


Figure 5 Classification of isotherms for adsorption from solution, according to Giles et al. (Ref. 132).

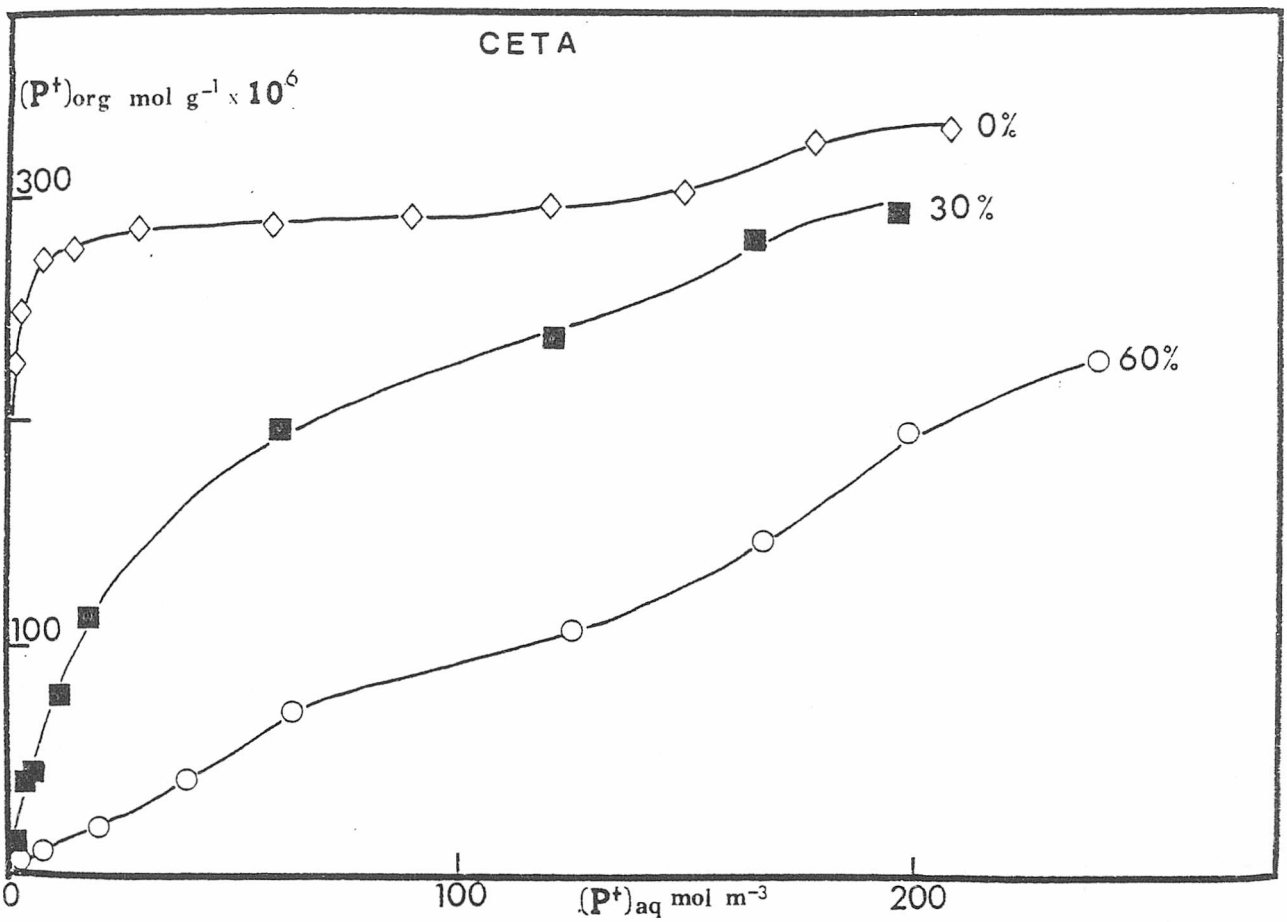


Figure 6a Adsorption isotherms for cetrimide from different concentrations of acetontrile–water mixtures.

◇– Pure water; ■– 30% v/v CH_3CN; ○– 60% v/v CH_3CN.

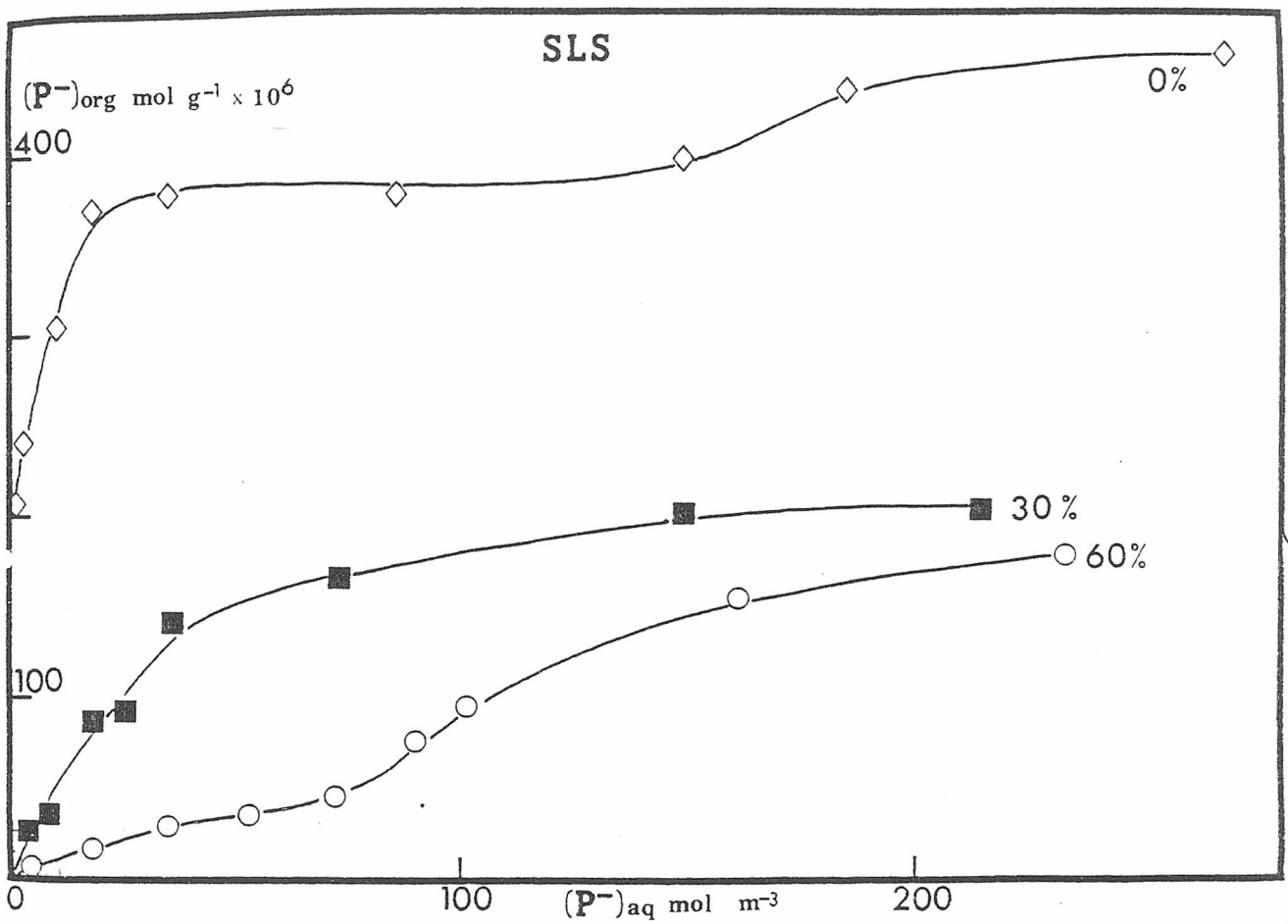


Figure 6b Adsorption isotherms for sodium laurylsulphate from different concentrations of acetonitrile-water mixtures.

◇- Pure water; ■- 30% v/v CH_3CN; ○- 60% v/v CH_3CN.

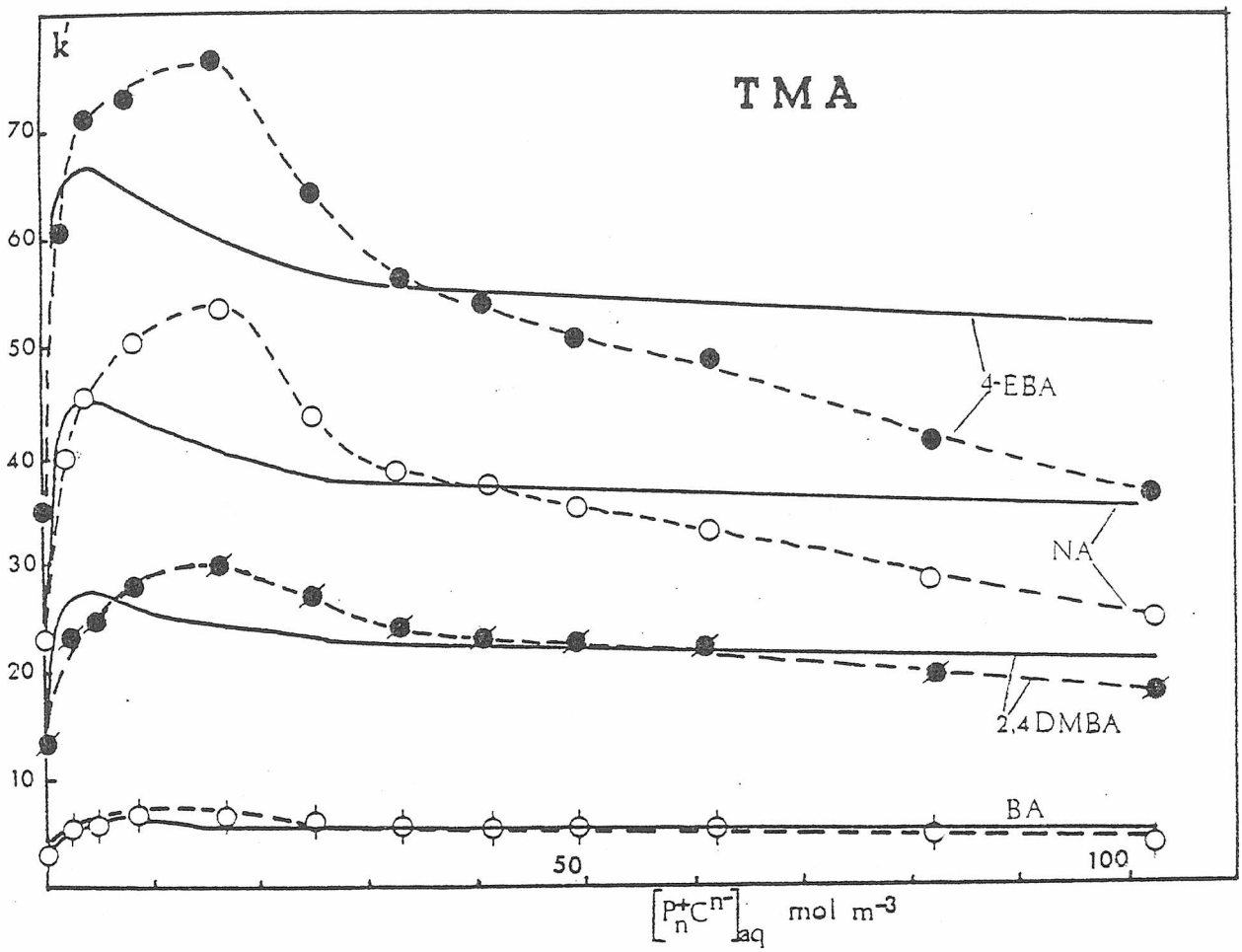


Figure 7a Plots showing the variation of capacity factor (k') with aqueous (TMA) concentration for the four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

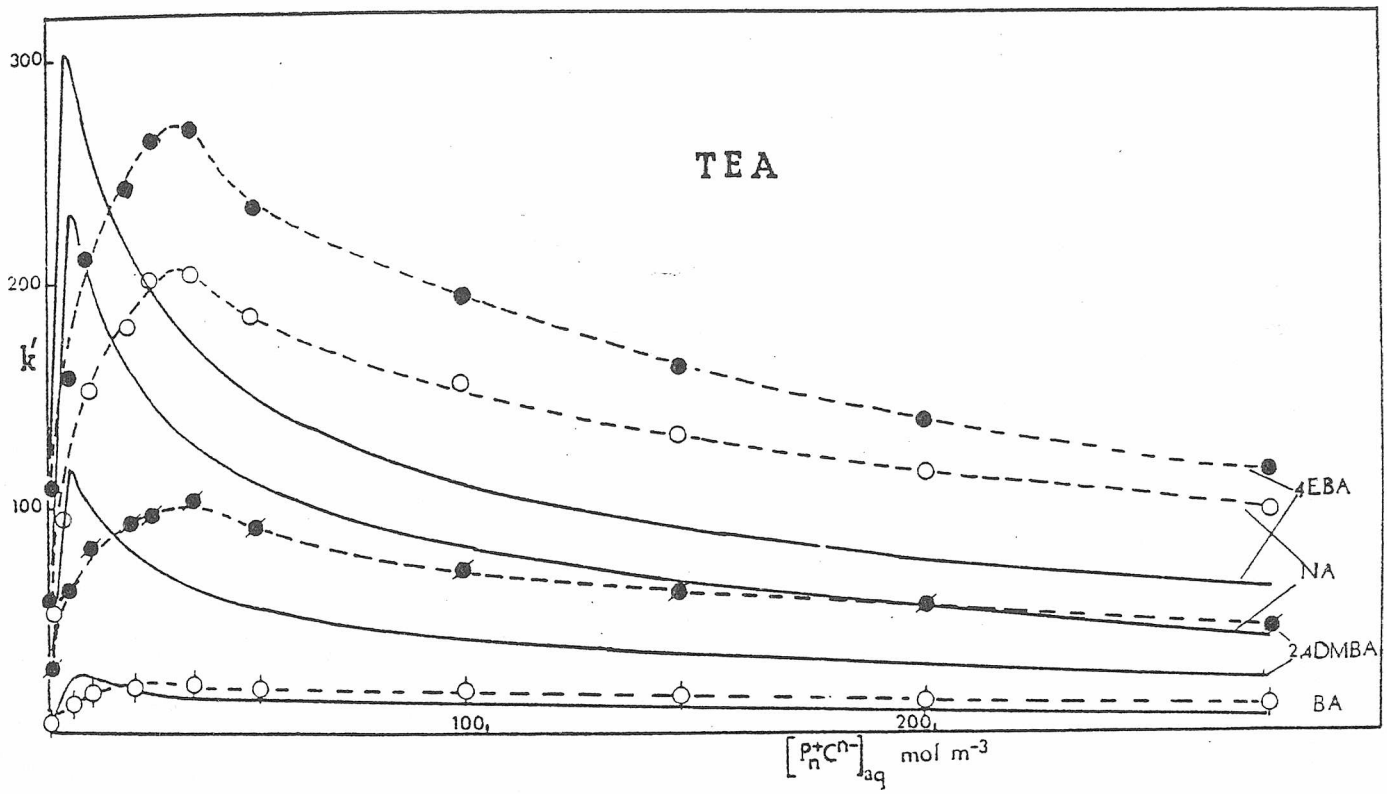


Figure 7b Plots showing the variation of capacity factor (k') with aqueous (TEA) concentration for the four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

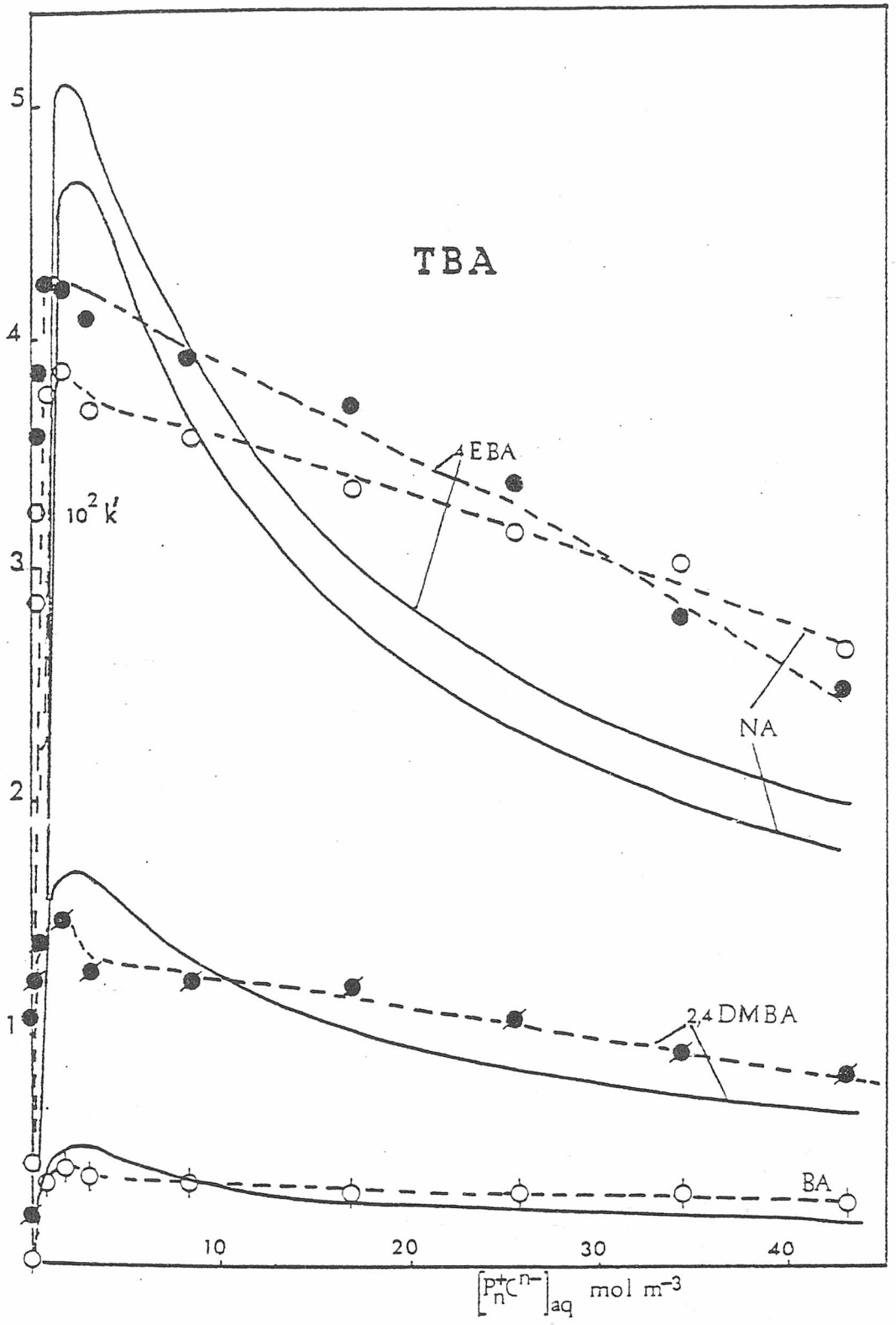


Figure 7c Plots showing the variation of capacity factor (k') with aqueous (TBA) concentration for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

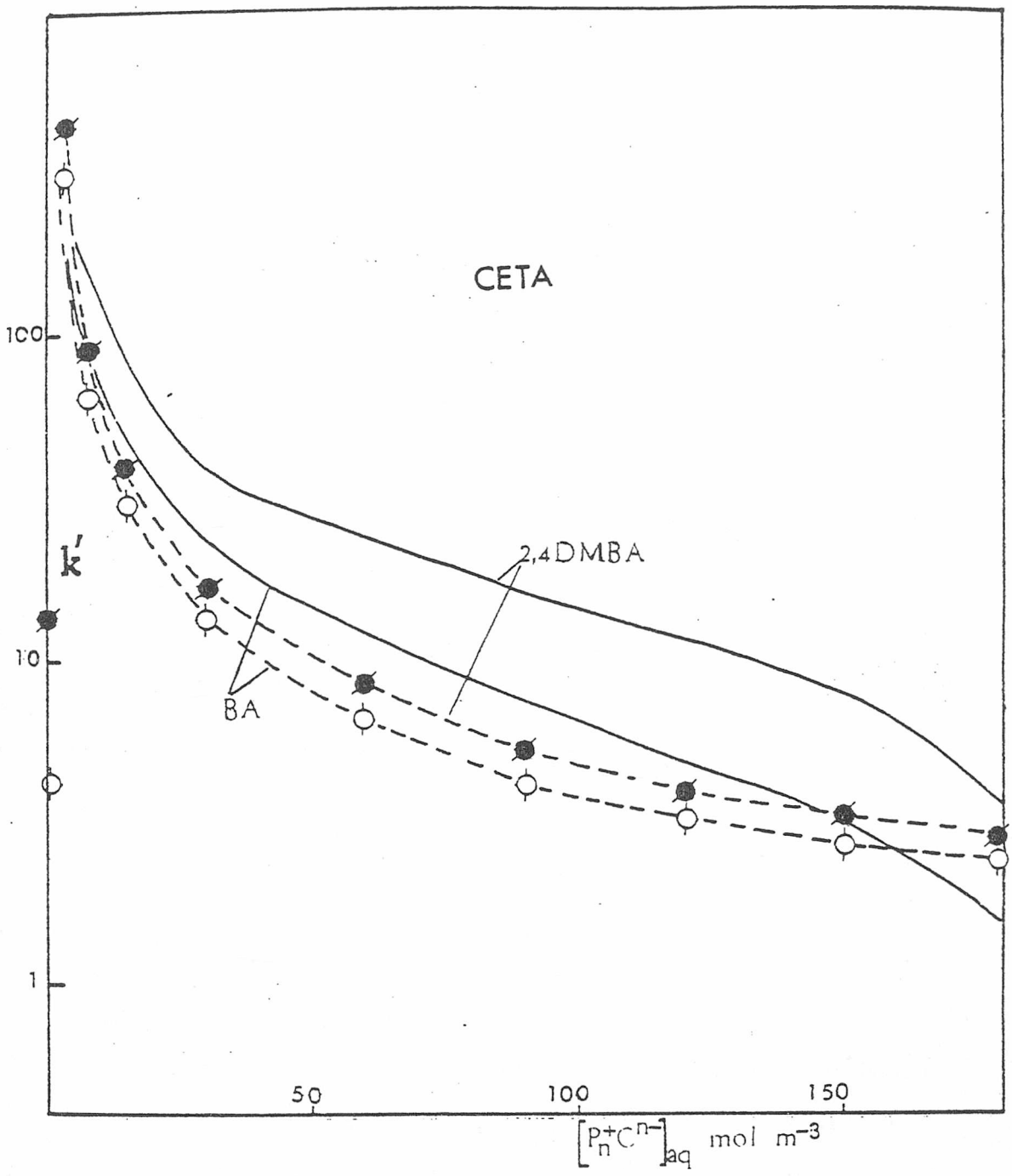


Figure 7d Plots showing the variation of capacity factor (k') with aqueous (CETA) concentration for two acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

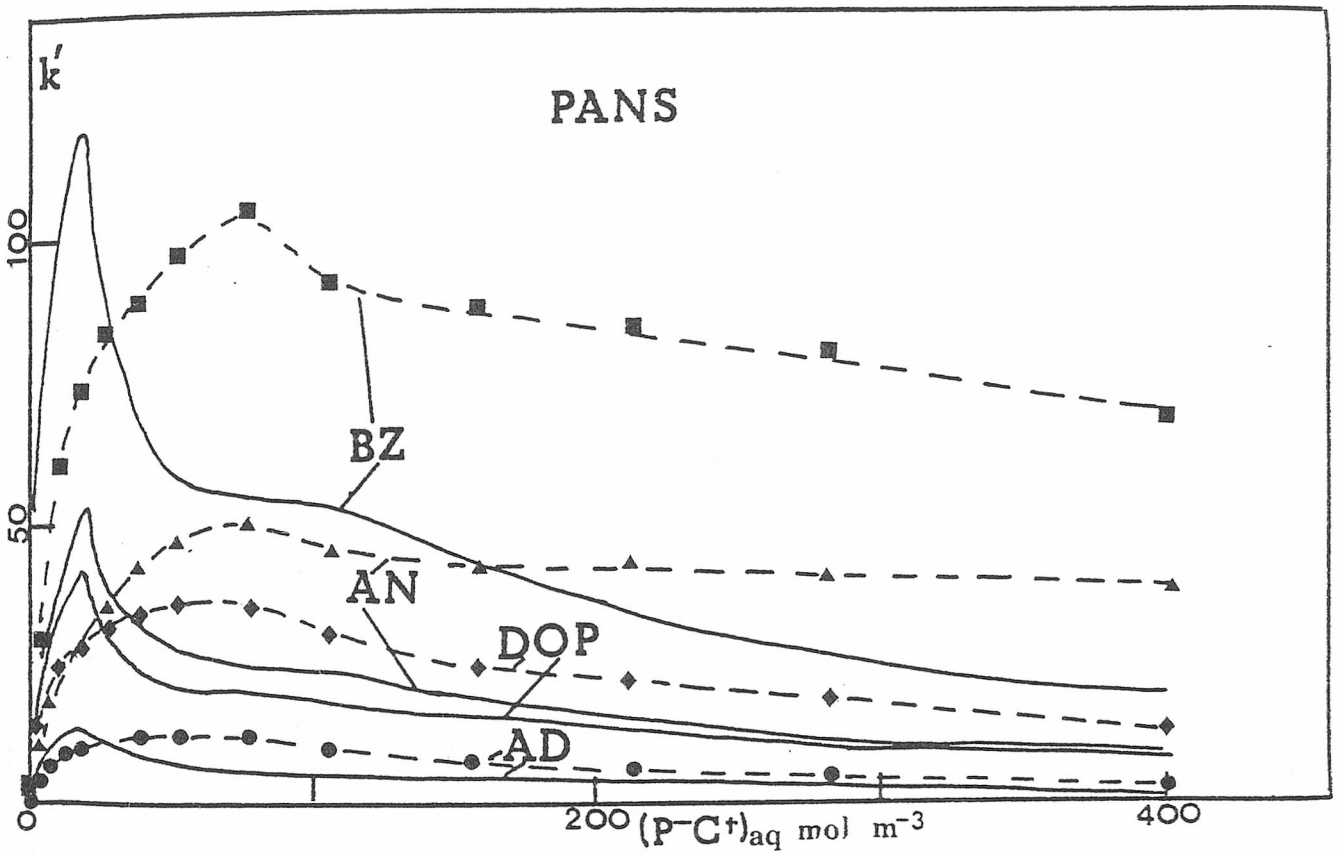


Figure 8a Plots showing the variation of capacity factor (k') with aqueous (PANS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

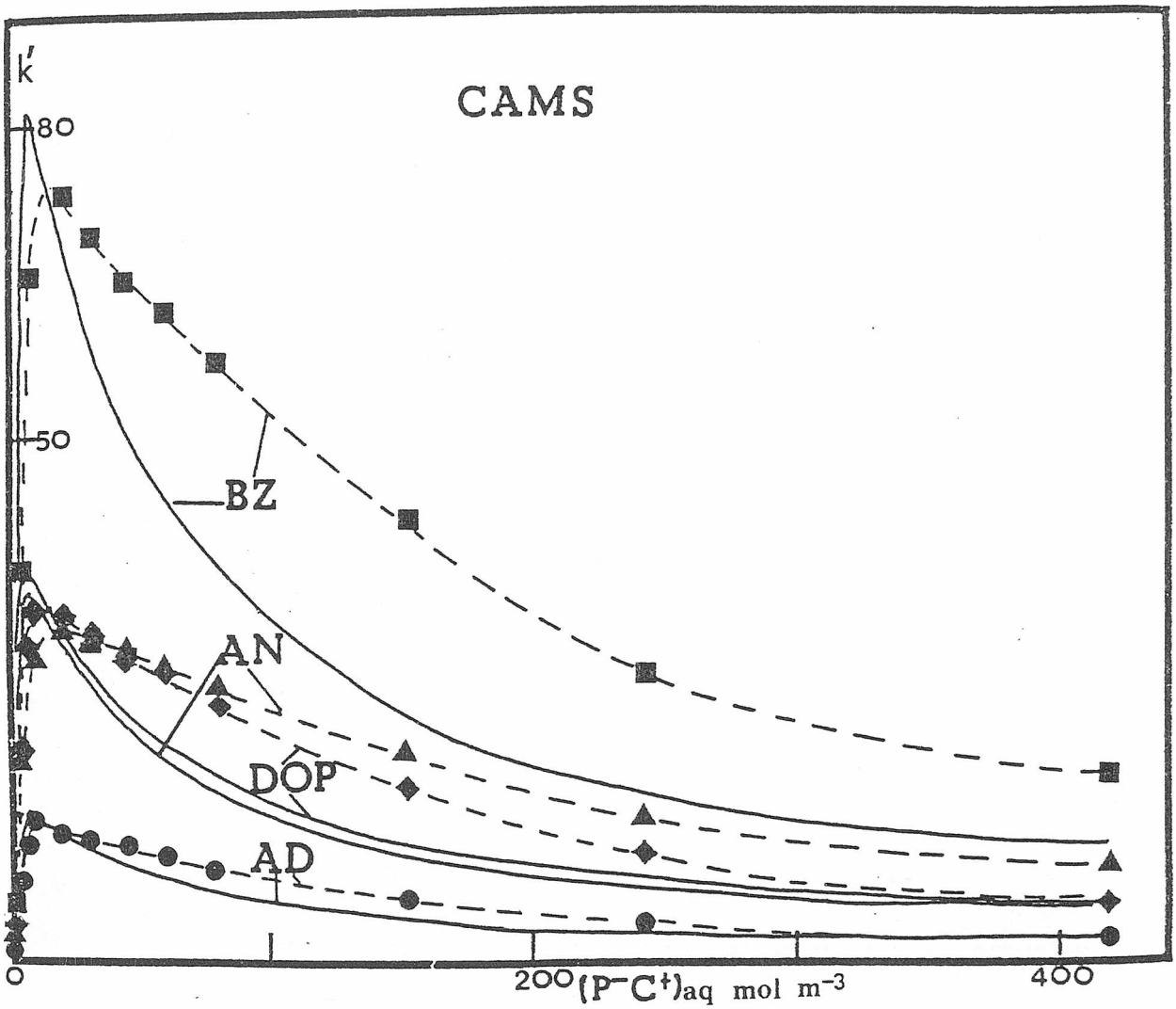


Figure 8b Plots showing the variation of capacity factor (k') with aqueous (CAMS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

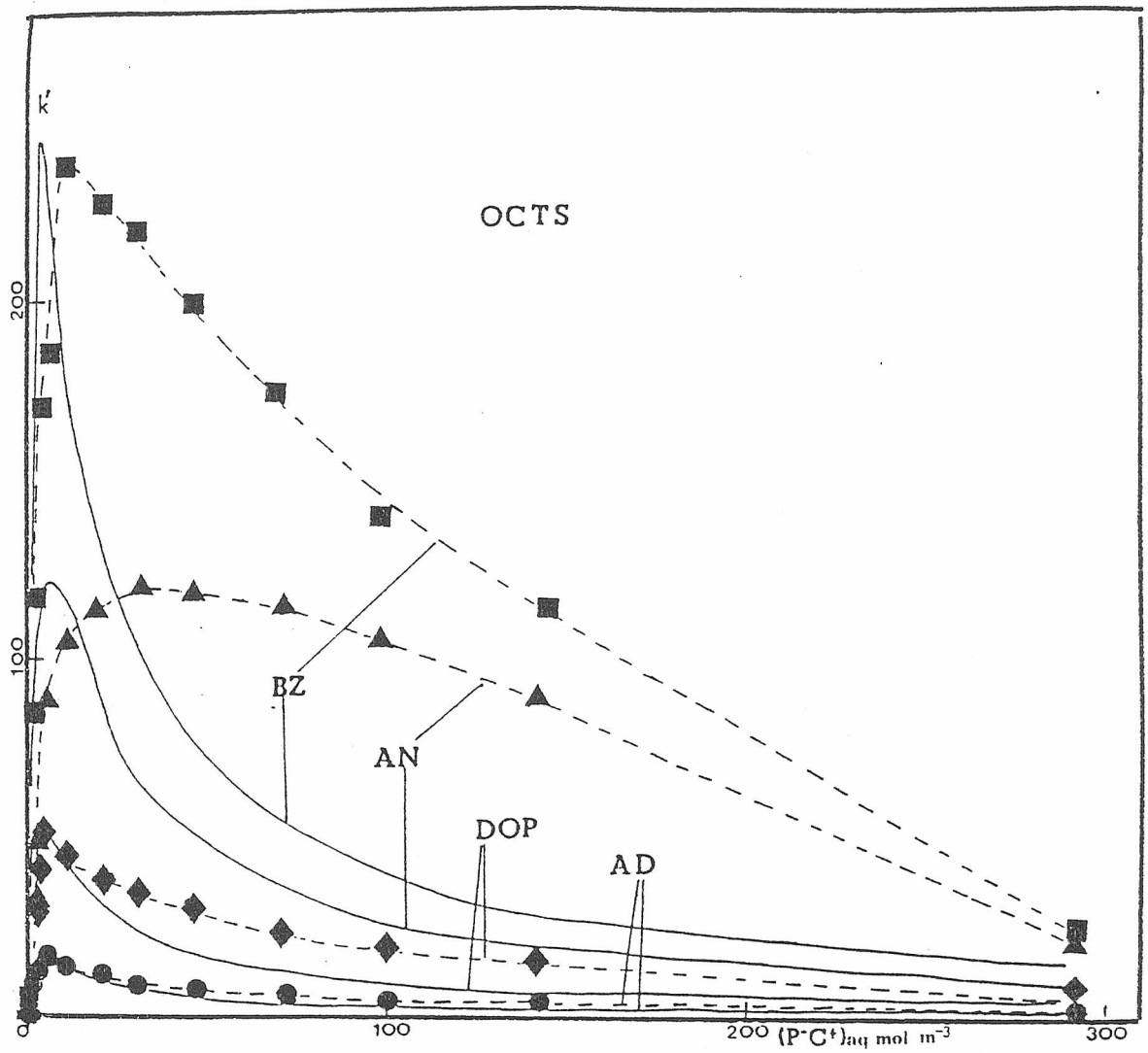


Figure 8c Plots showing the variation of capacity factor (k') with aqueous (OCTS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

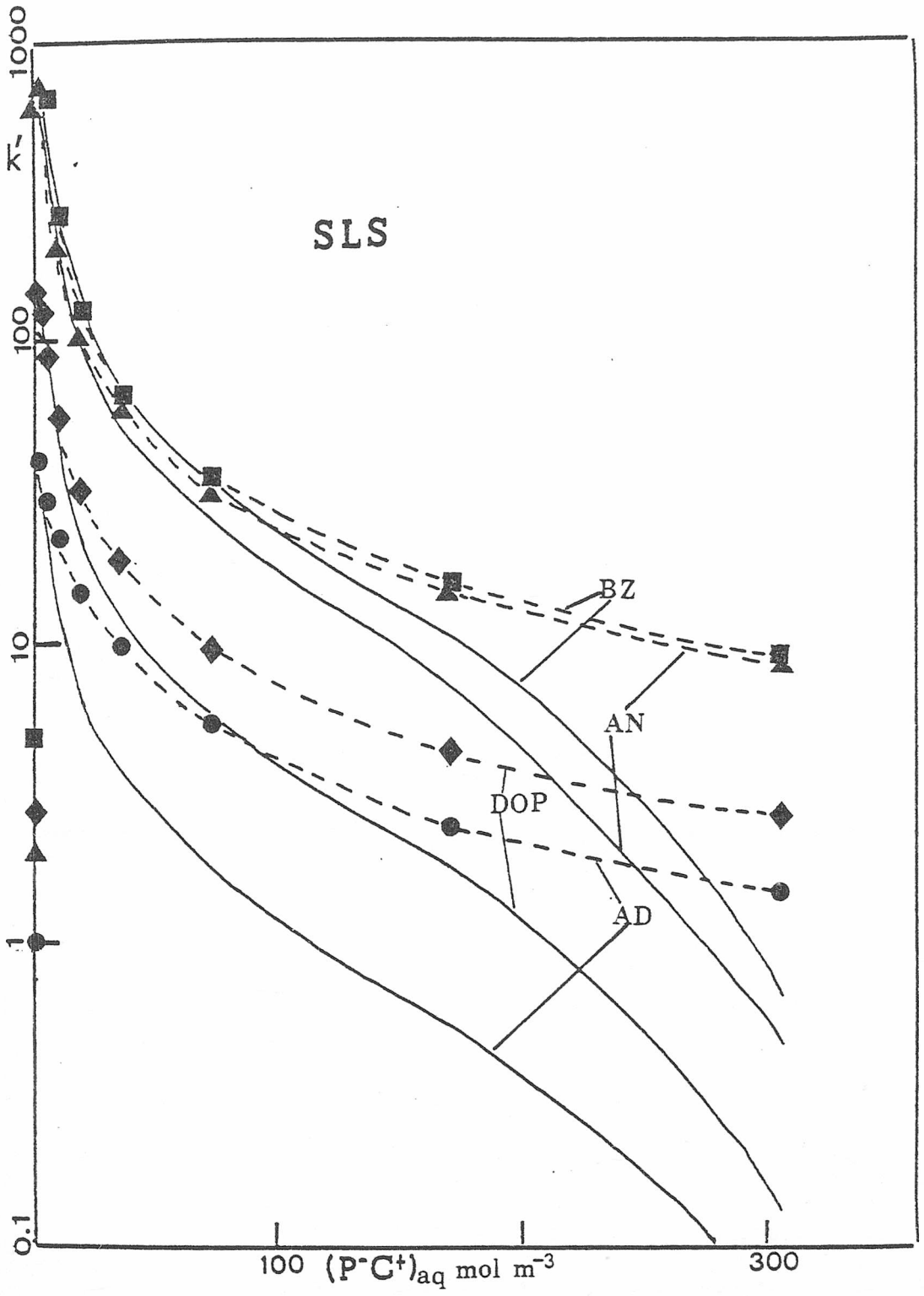


Figure 8d Plots showing the variation of capacity factor (k') with aqueous (SLS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

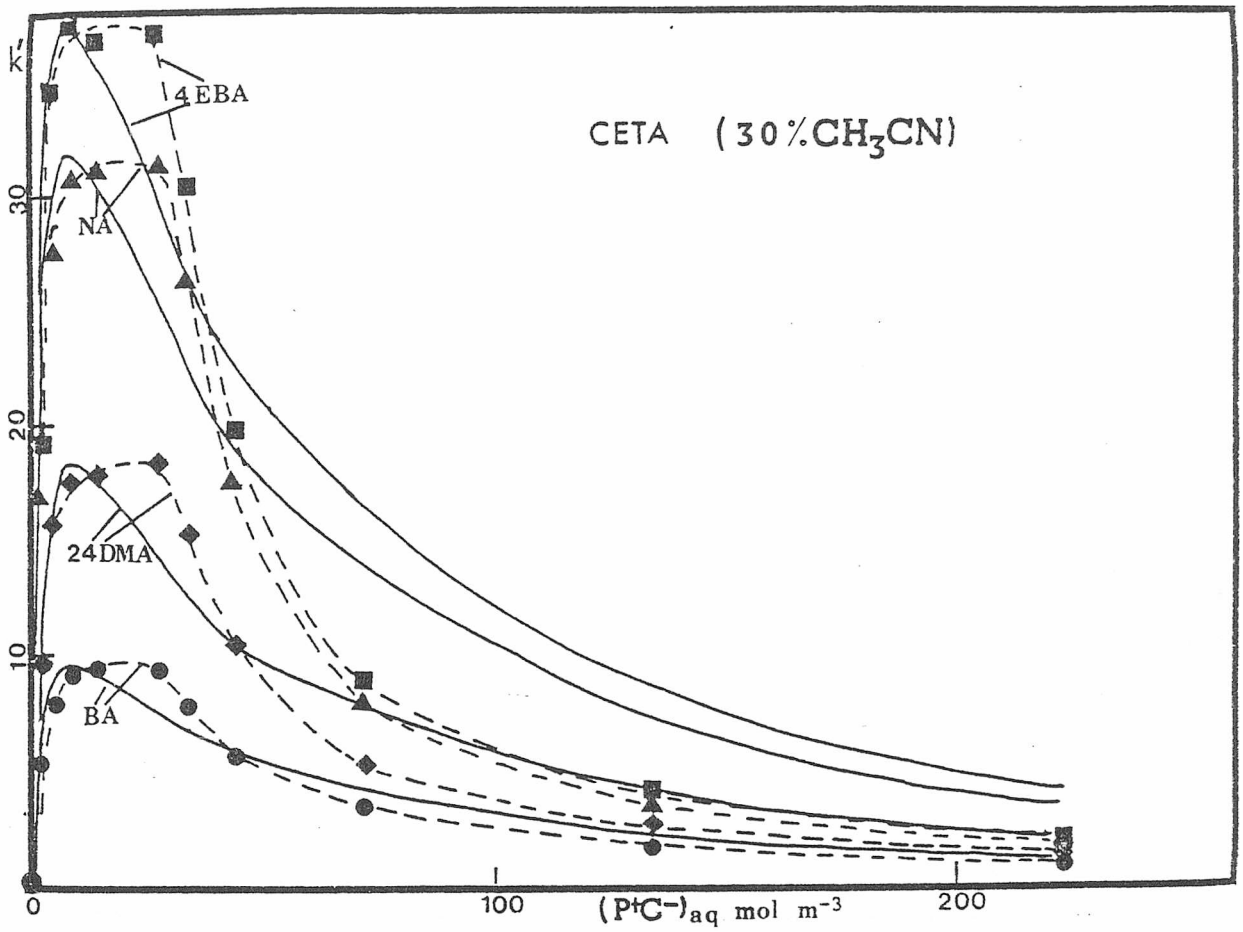


Figure 9a Plots showing the variation of capacity factor (k') with (CETA) concentration in 30% v/v acetonitrile-water mixture for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

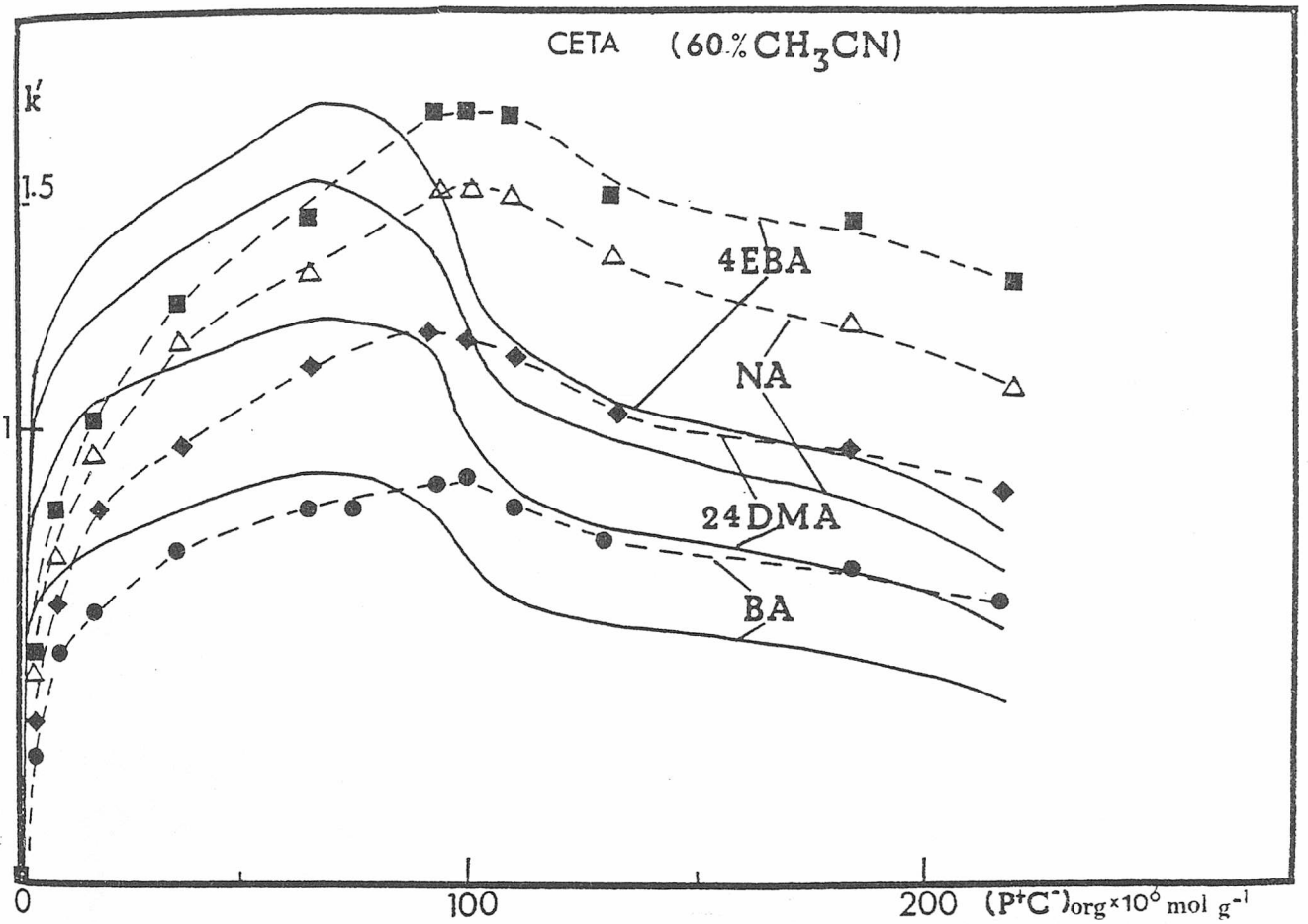


Figure 9b Plots showing the variation of capacity factor (k') with (CETA) concentration in 60% v/v acetonitrile–water mixture for four acidic solutes. Dashes lines represent experimental results. Full lines represent curves calculated from Equation (45).

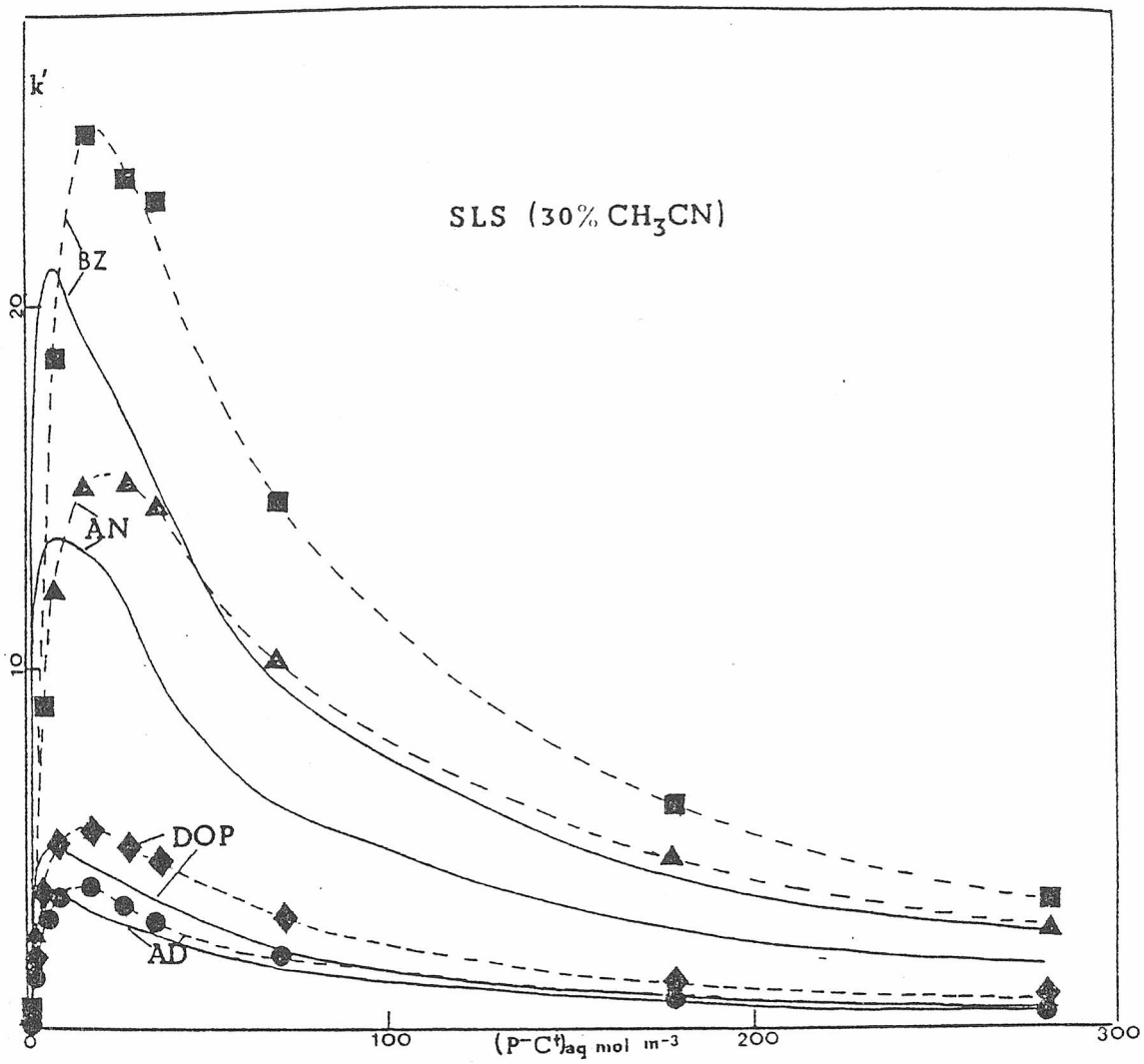


Figure 10a Plots showing the variation of capacity factor (k') with (SLS) concentration in 30% v/v acetonitrile–water for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

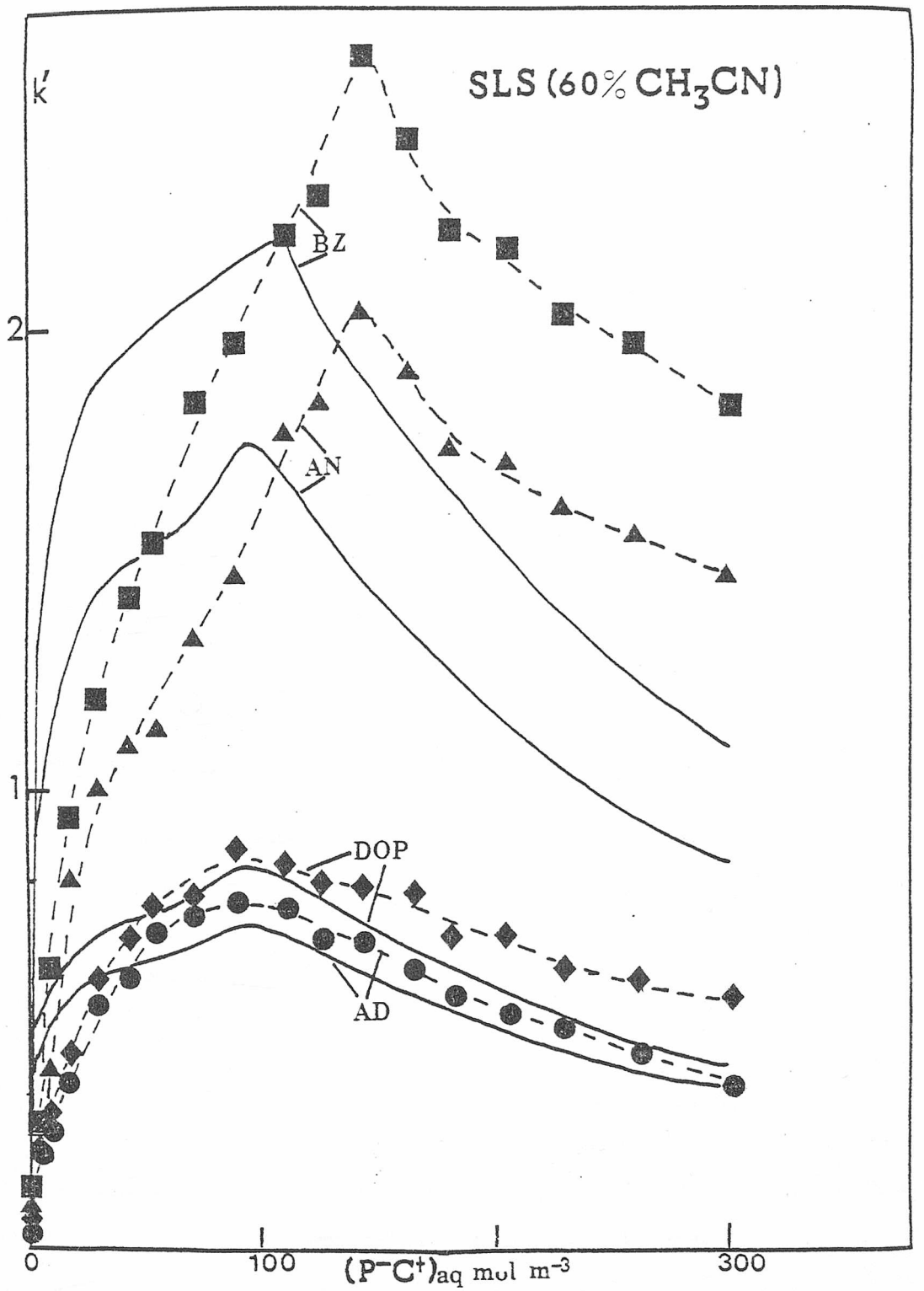


Figure 10b Plots showing the variation of capacity factor (k') with (SLS) concentration in 60% v/v acetonitrile-water mixture for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

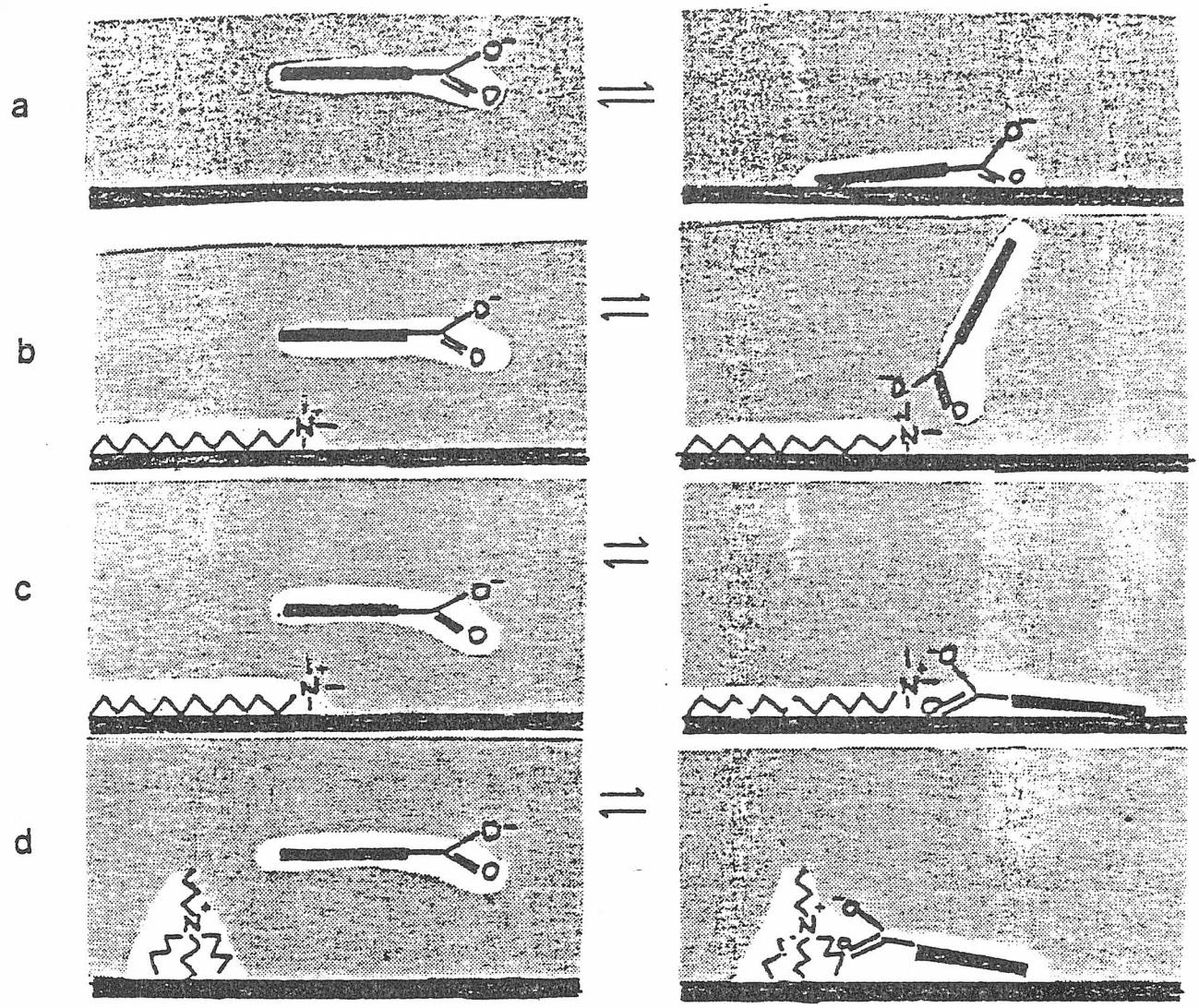


Figure 11 Schematic representation of the postulated retention mechanisms discussed in the text.

(a) desolvation of an ionised solute;

(b) ion exchange involving an adsorbed pairing ion without subsequent desolvation of solute;

(c) and (d) ion exchange reinforcing desolvation of solute for adsorbed surfactant (c) and non-surfactant (d) pairing ions.

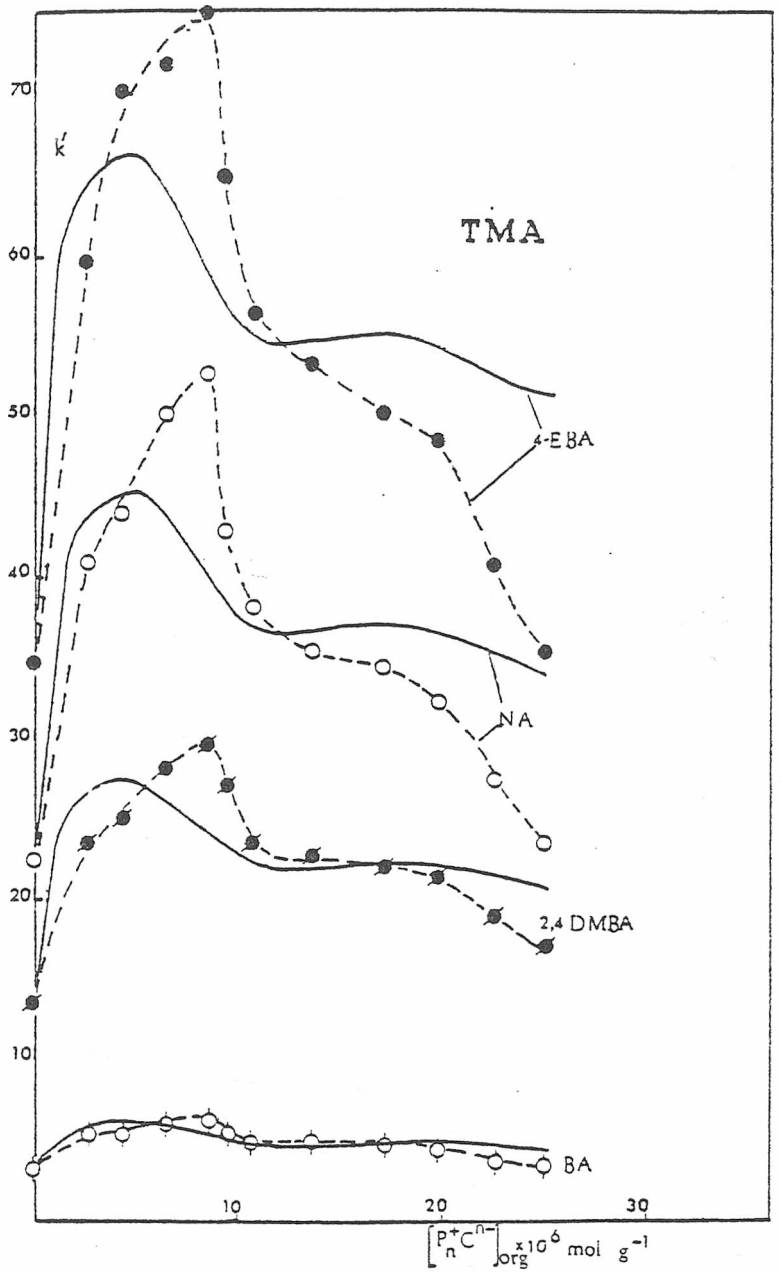


Figure 12a Plots showing the variation of capacity factor (k') with adsorbed (TMA) concentration for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

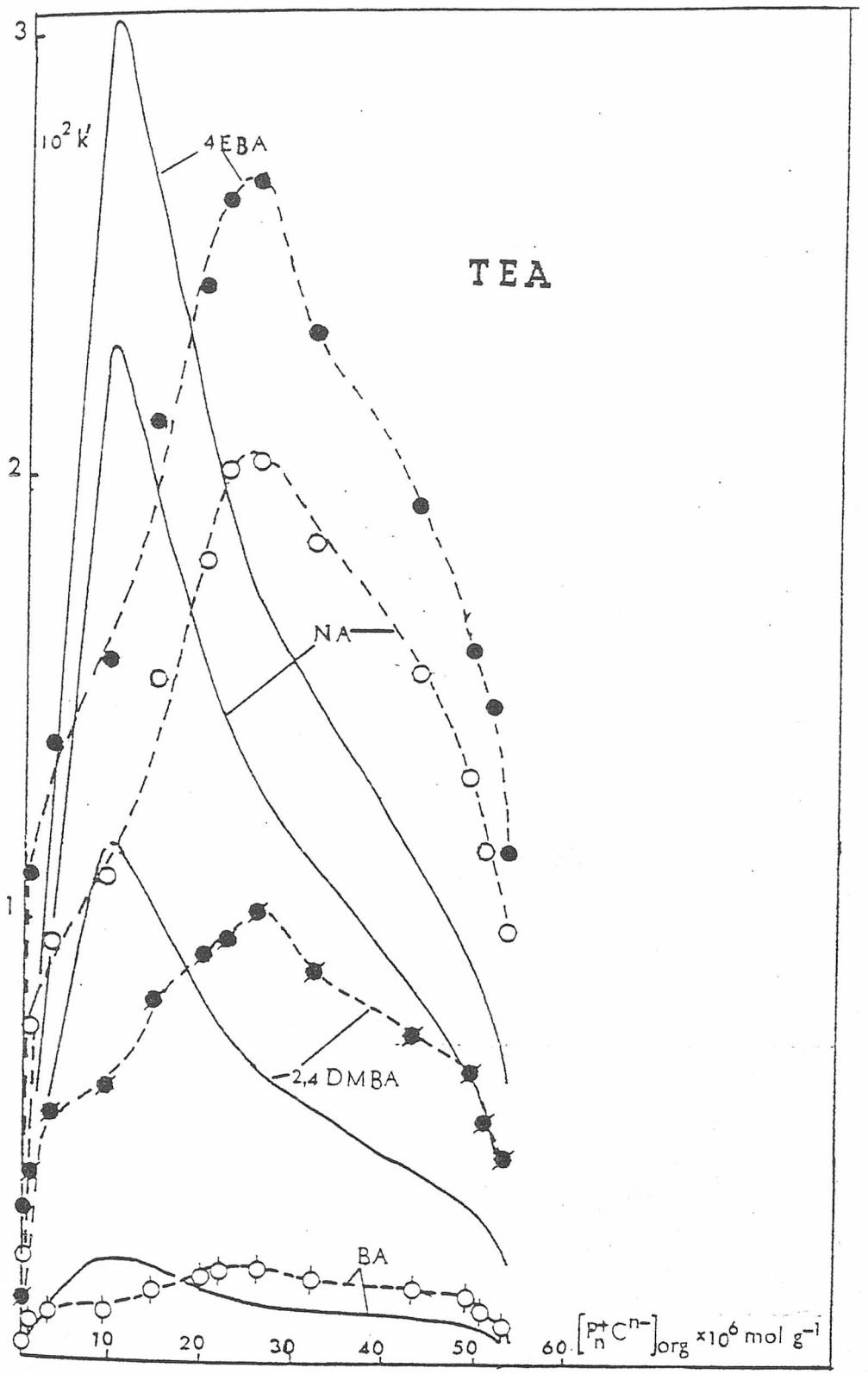


Figure 12b Plots showing the variation of capacity factor (k') with adsorbed (TEA) concentration for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

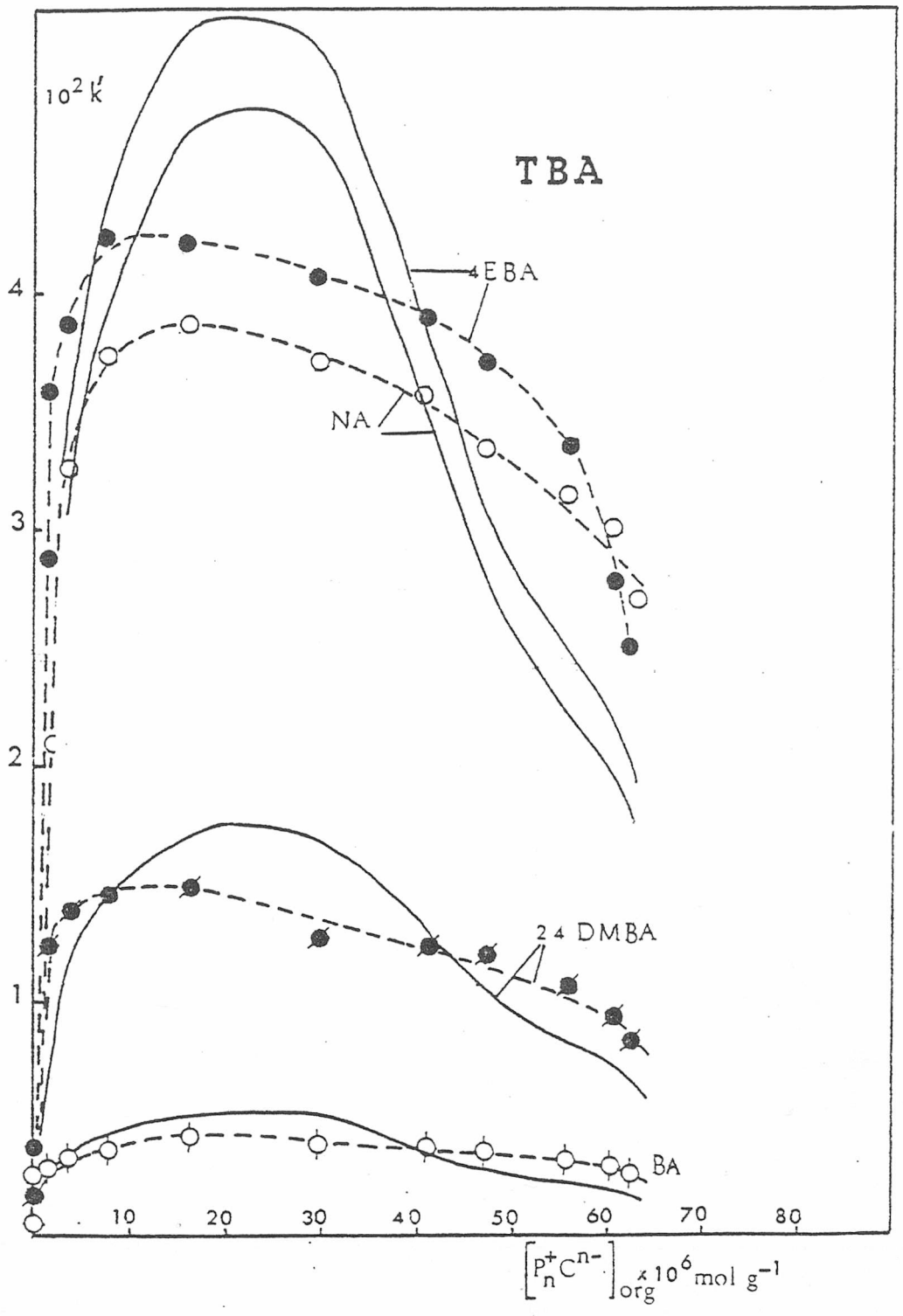


Figure 12c Plots showing the variation of capacity factor (k') with adsorbed (TBA) concentration for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

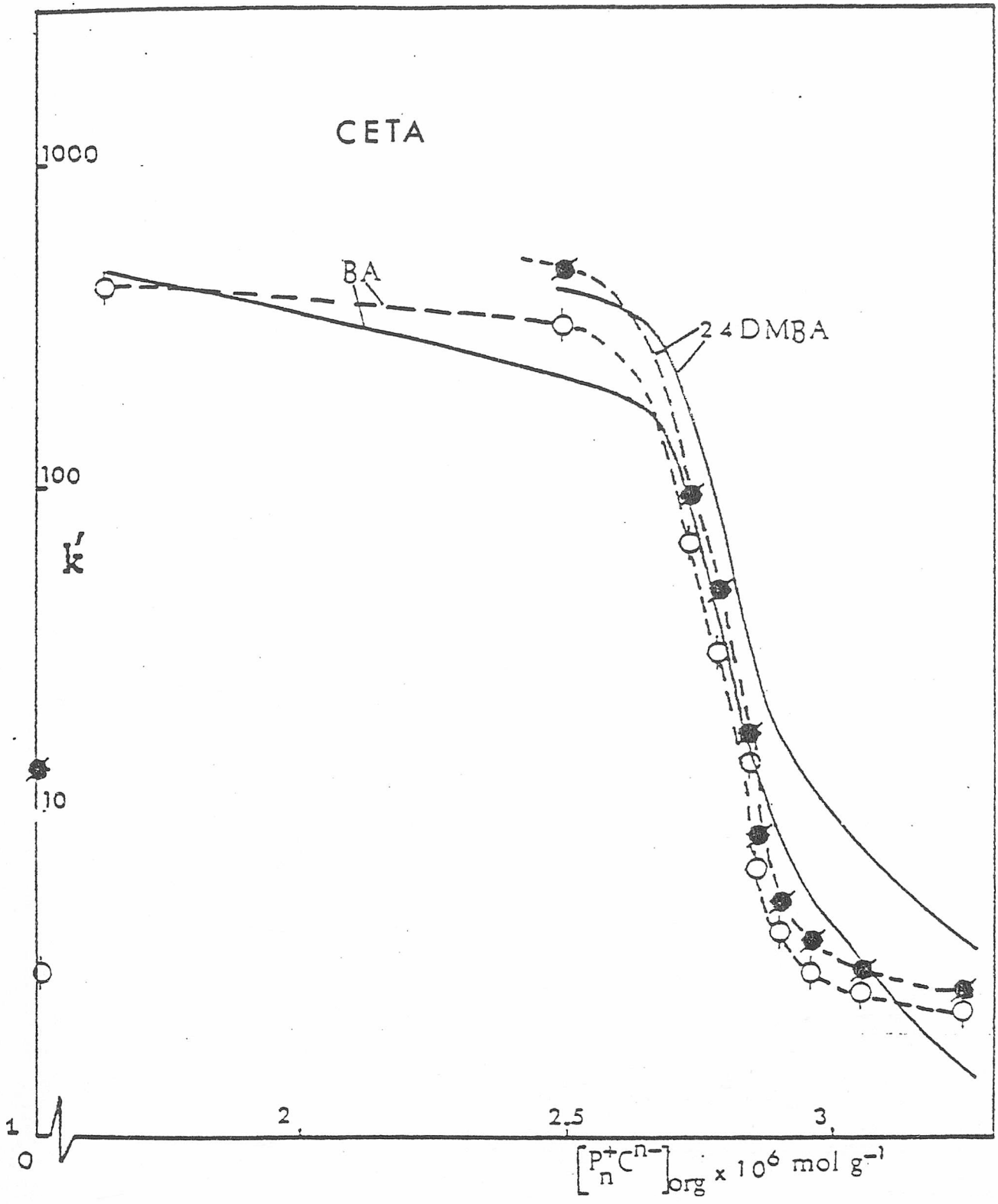


Figure 12d Plots showing the variation of capacity factor (k') with adsorbed (CETA) concentration for two acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

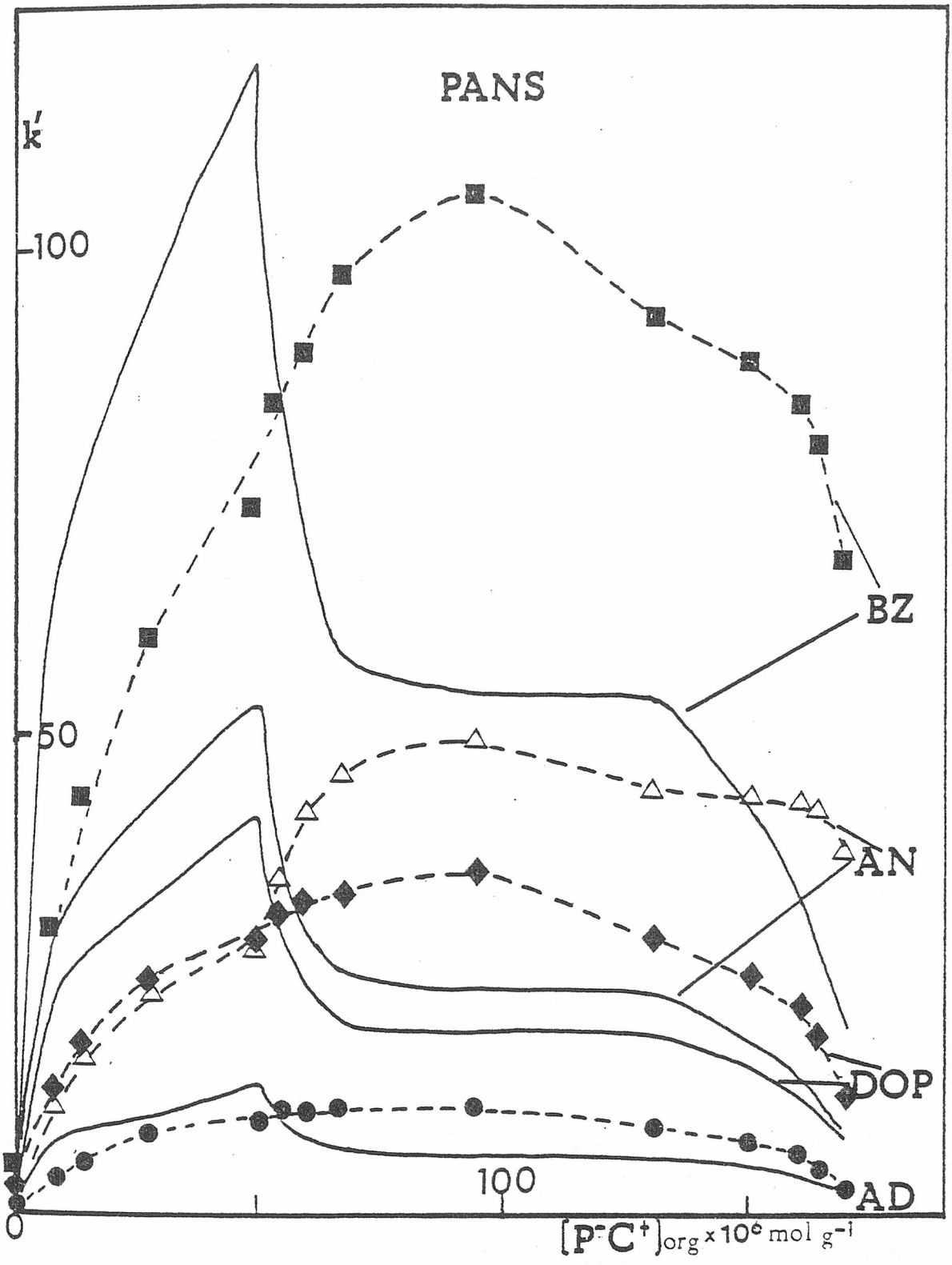


Figure 13a Plots showing the variation of capacity factor (k') with adsorbed (PANS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

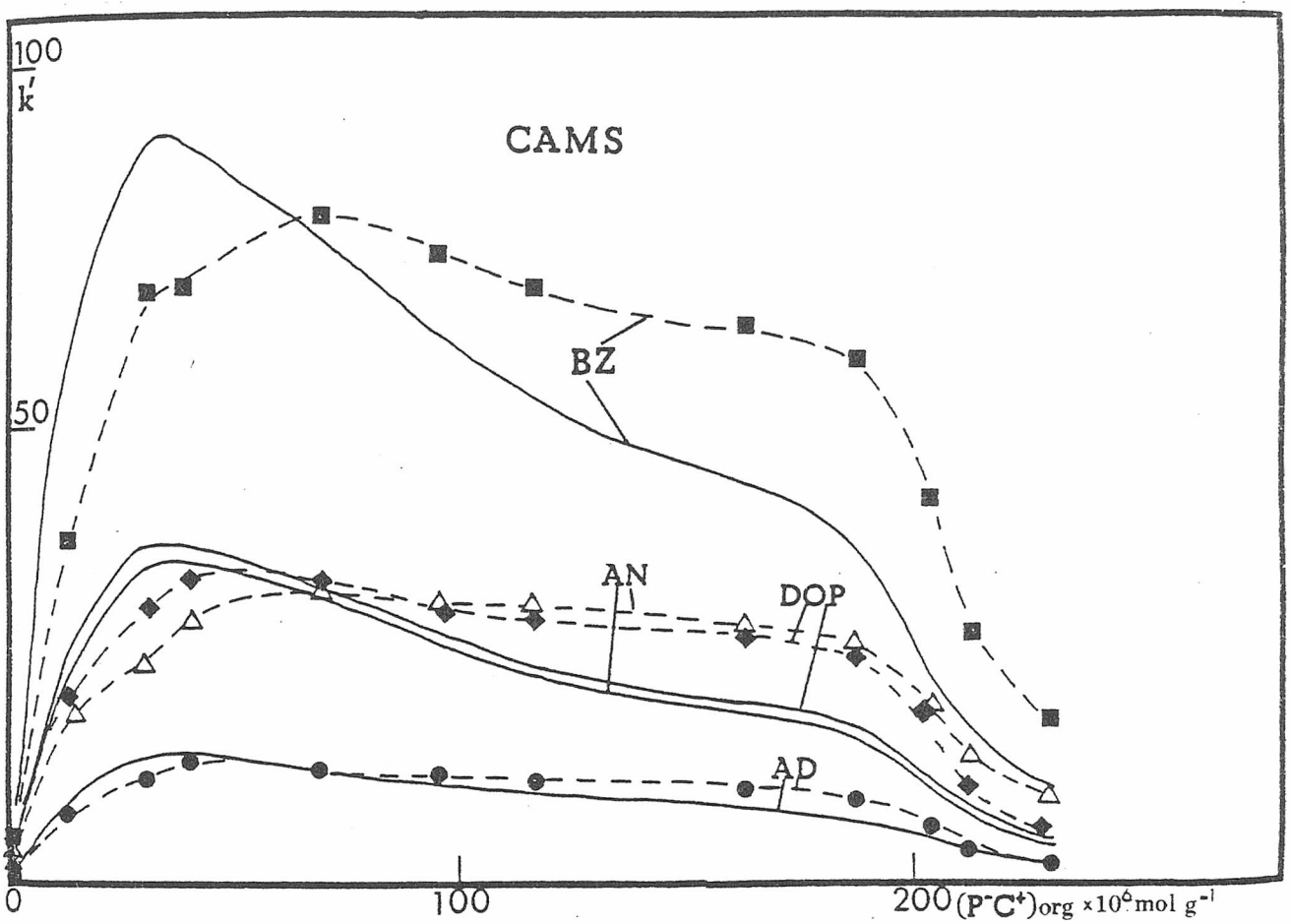


Figure 13b Plots showing the variation of capacity factor (k') with adsorbed (CAMS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

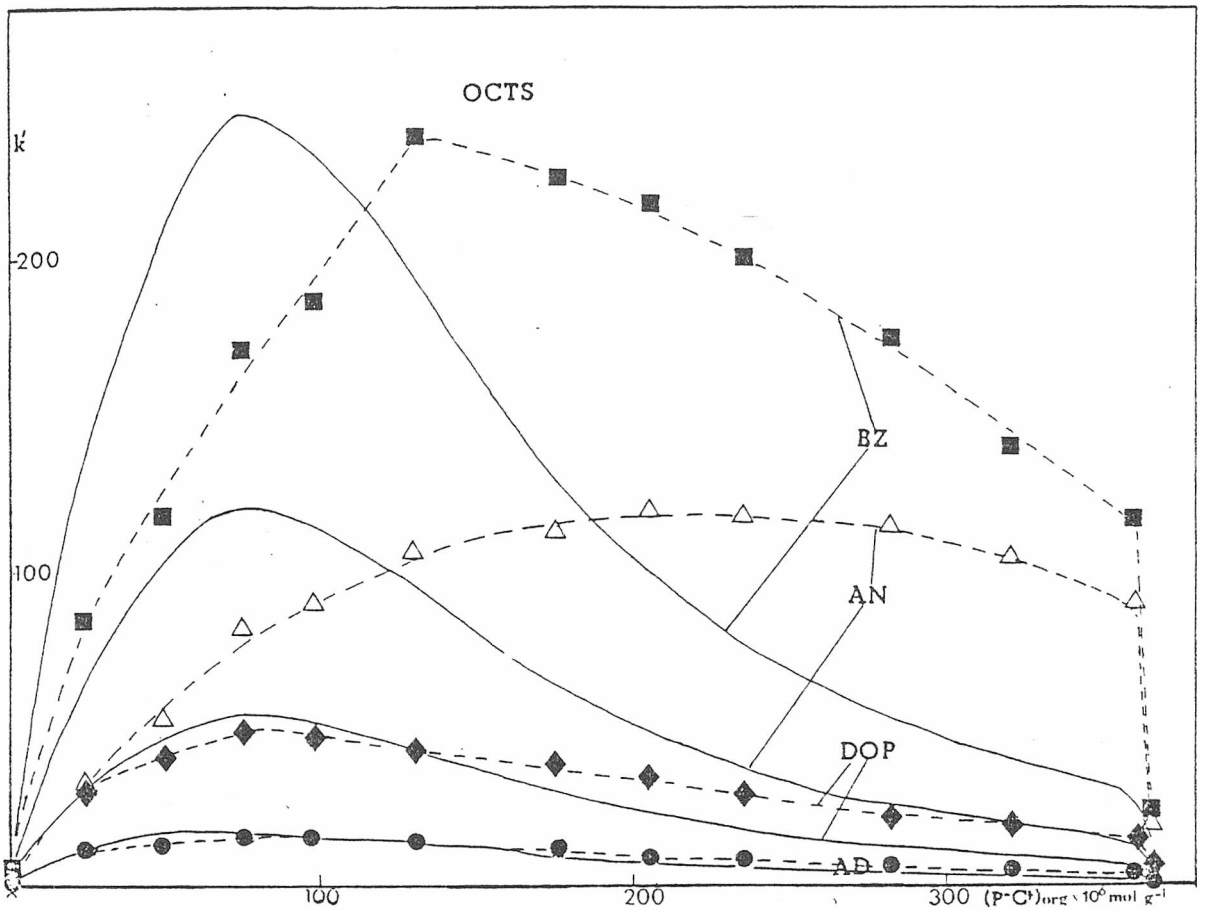


Figure 13c Plots showing the variation of capacity factor (k') with adsorbed (OCTS) concentration for four basic solutes. Dashes lines represent experimental results. Full lines represent curves calculated from Equation (45).

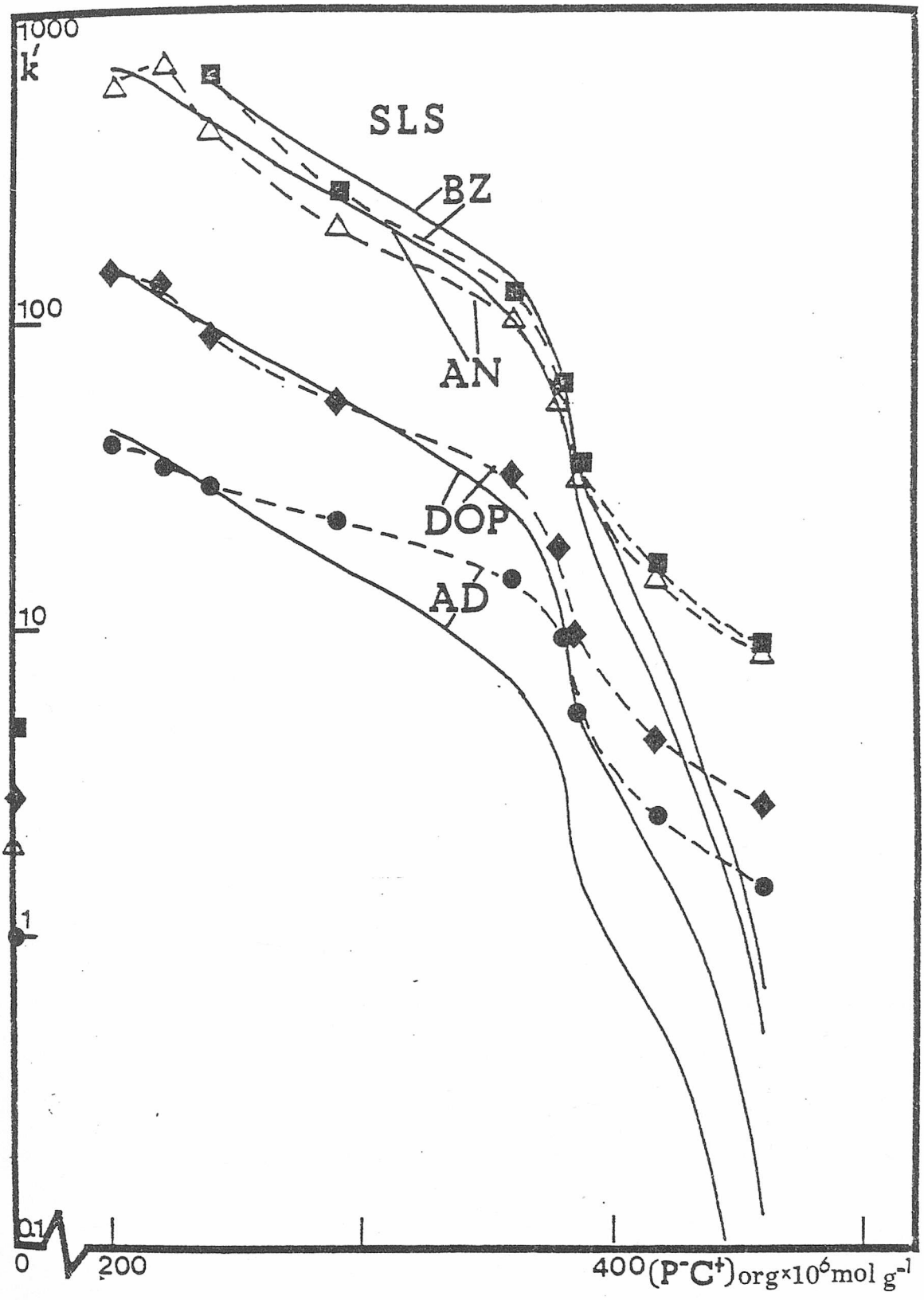


Figure 13d Plots showing the variation of capacity factor (k') with adsorbed (SLS) concentration for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

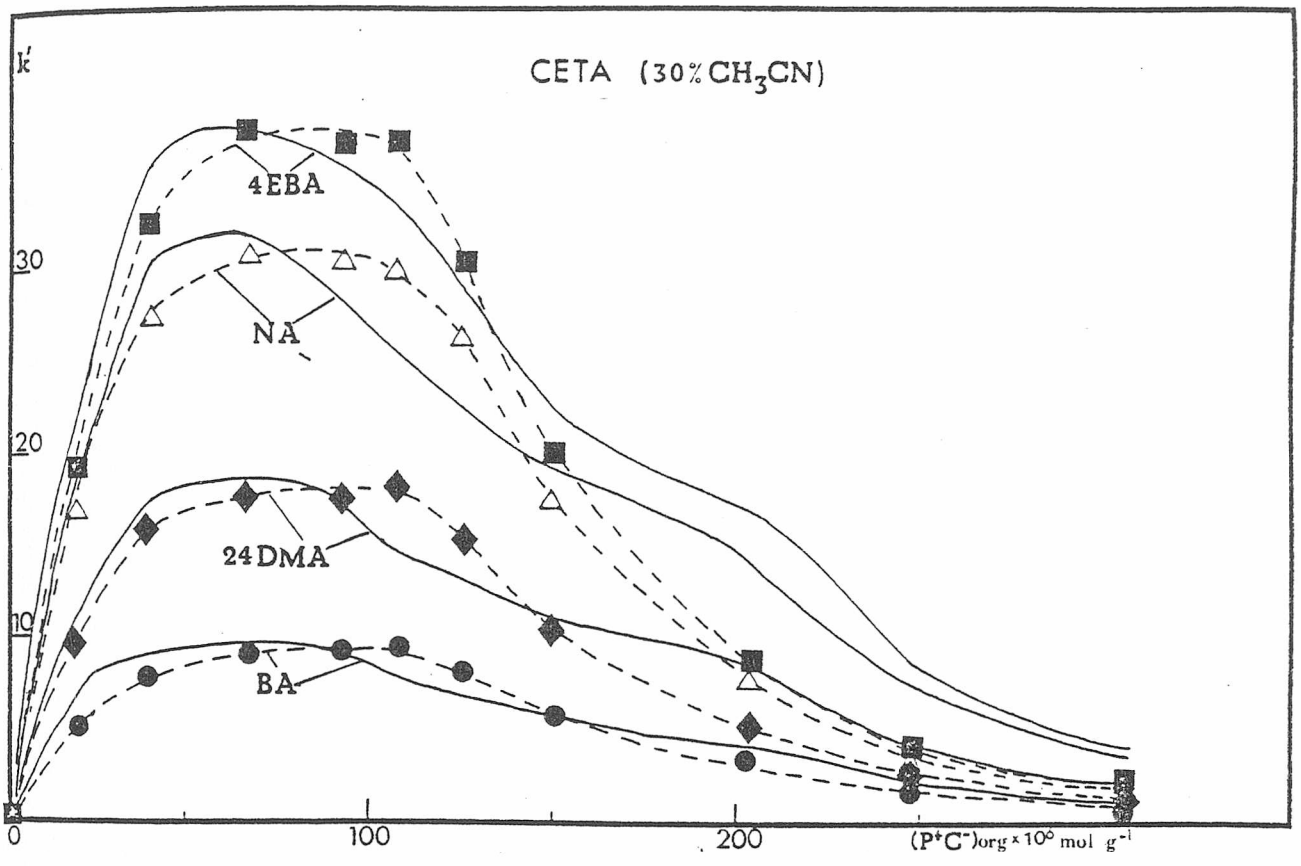


Figure 14a Plots showing the variation of capacity factor (k') with adsorbed (CETA) concentration in mobile phase of 30% v/v acetonitrile–water mixtures for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

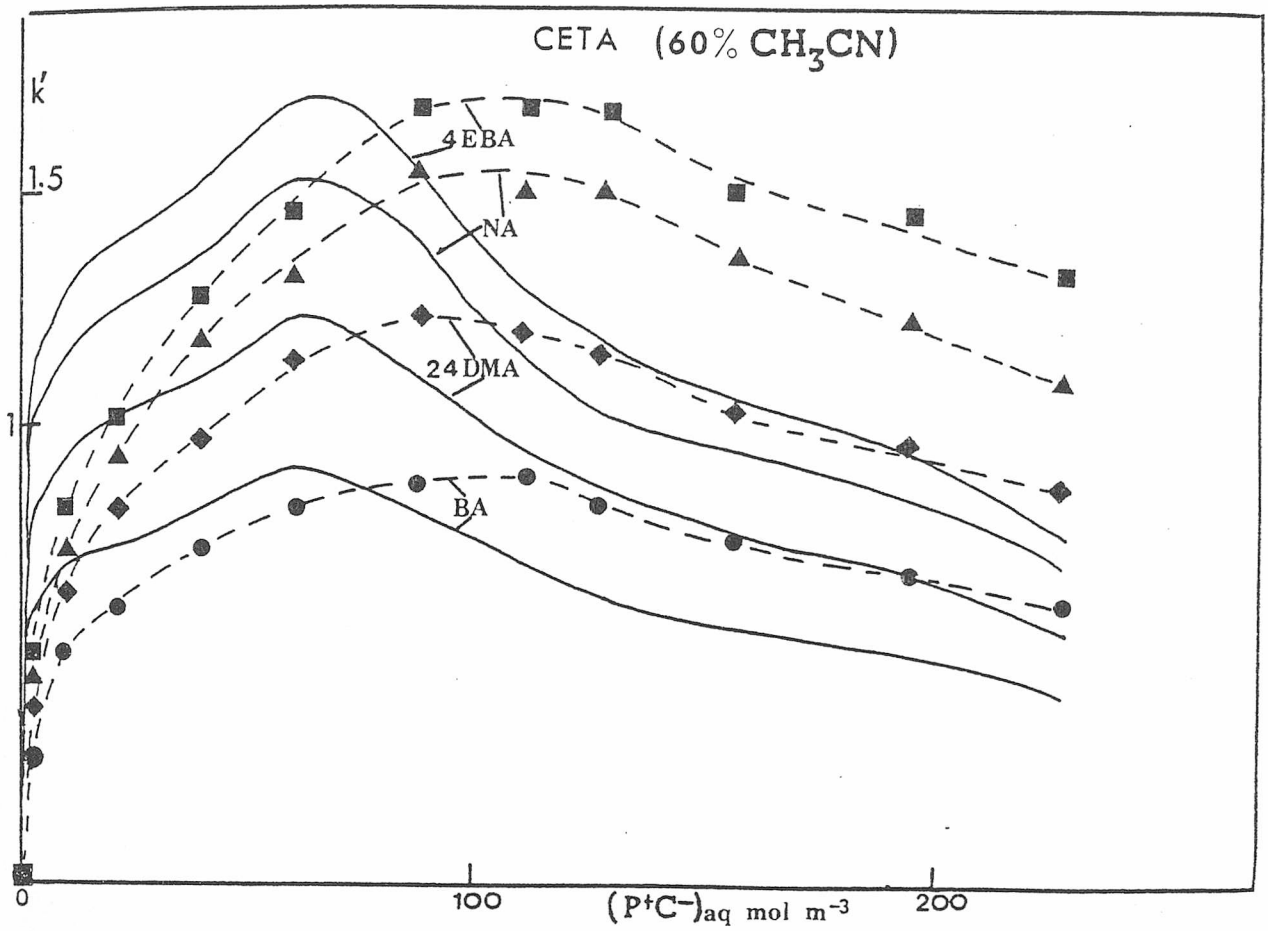


Figure 14b Plots showing the variation of capacity factor (k') with adsorbed (CETA) concentration in mobile phase of 60% v/v acetonitrile–water mixtures for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

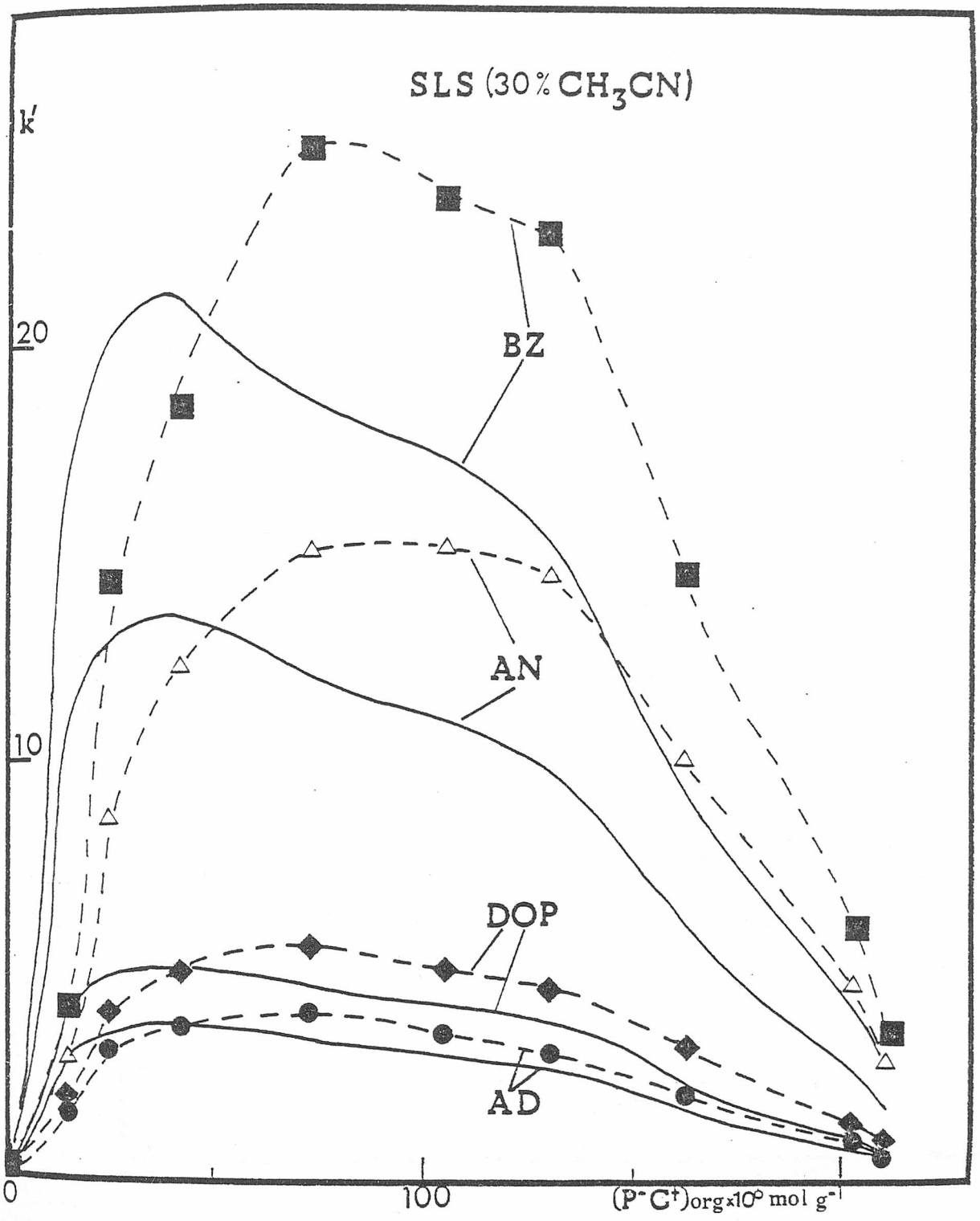


Figure 15a Plots showing the variation of capacity factor (k') with adsorbed (SLS) concentration in mobile phase of 30% v/v acetonitrile–water mixtures for four acidic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

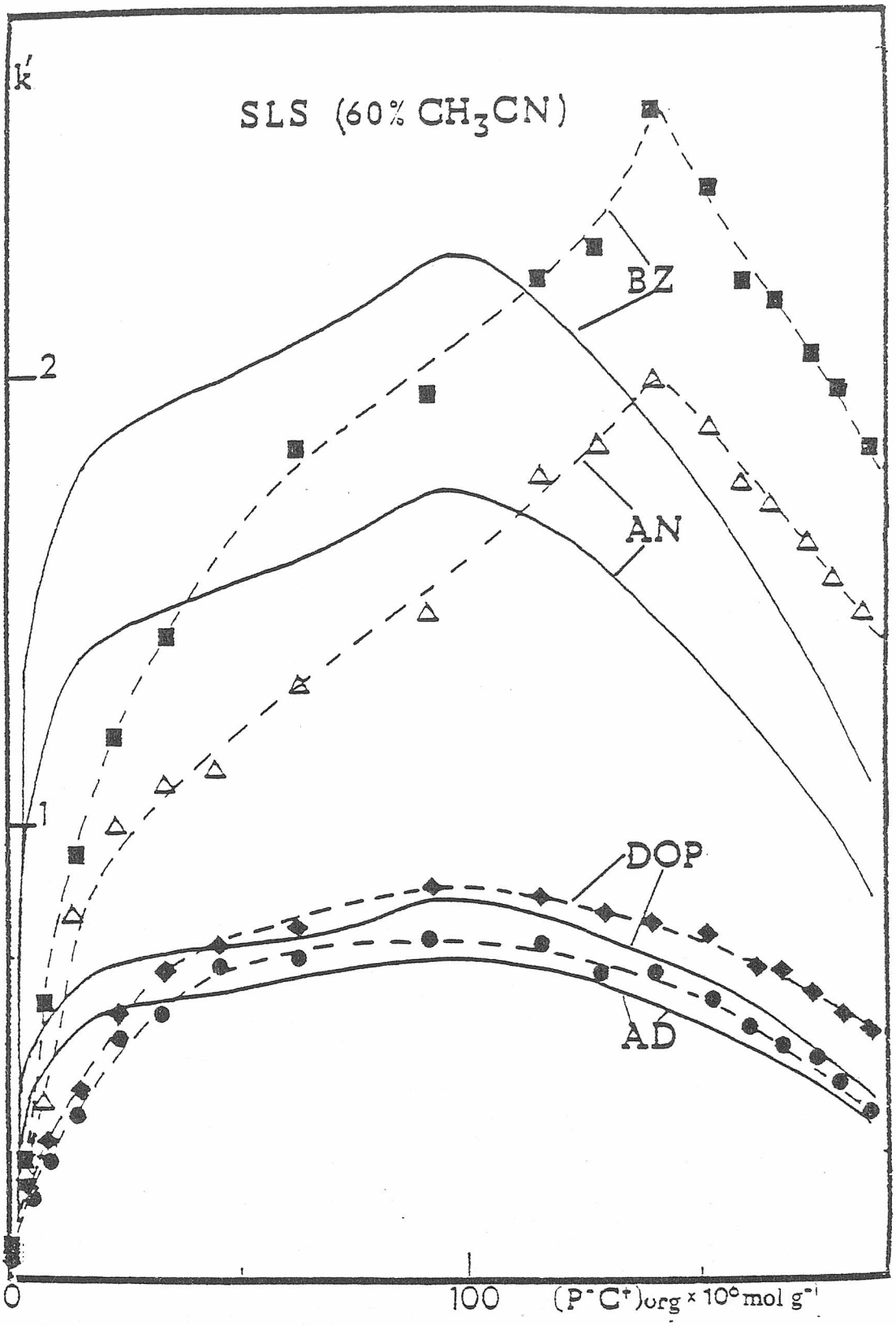


Figure 15b Plots showing the variation of capacity factor (k') with adsorbed (SLS) concentration in mobile phase of 60% v/v acetonitrile–water mixtures for four basic solutes. Dashed lines represent experimental results. Full lines represent curves calculated from Equation (45).

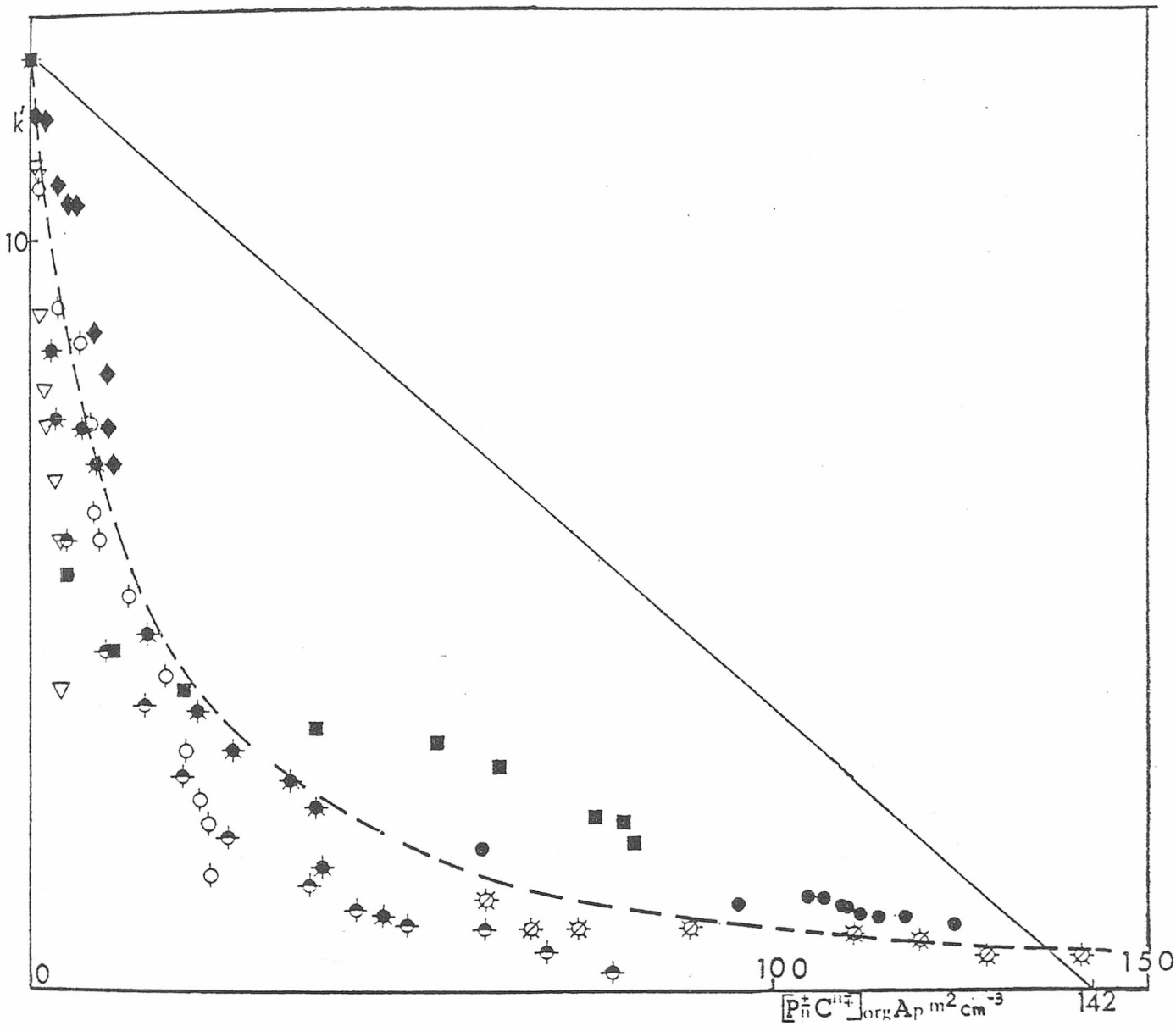
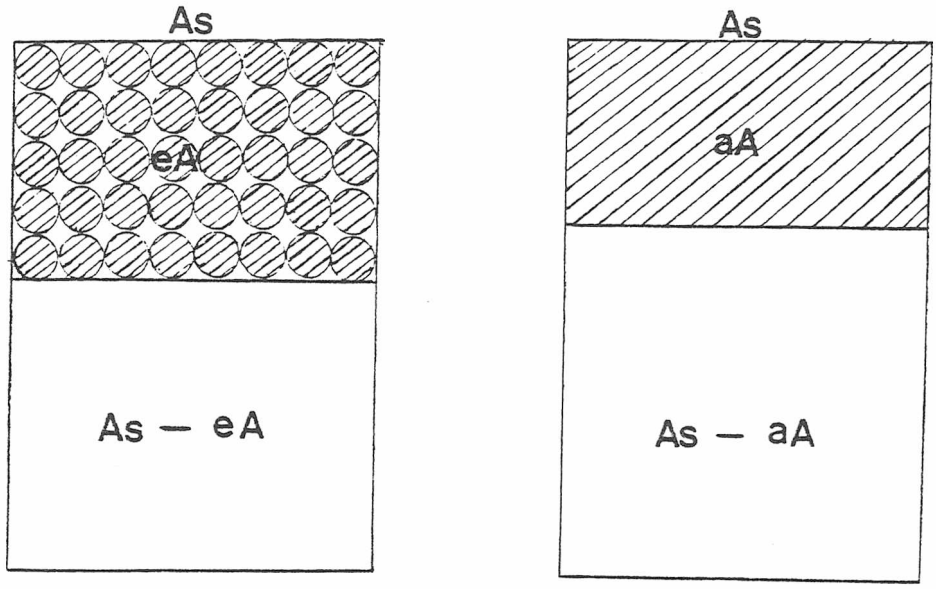


Figure 16 Plot showing the variation of capacity factor of 5HMF as a function of the area of the C-18 calculated as being covered by various pairing ions at the concentrations studied. Dashed line represents experimental results. Full line represents Equation (48).

▽- TMA; ◆- TEA; □- TBA; ●- CETA; ◇- PANS; ✱- CAMS; ✲- OCTS; ✲- SLS.



◍ - Pairing ion;

AS- Total surface area of the C-18 silica available for desolvation of the solutes in the absence of pairing ion;

eA- Total effective surface area occupied by the adsorbed pairing ion on the C-18 surface;

aA- Actual surface area occupied by the pairing ion on the C-18 surface.

Figure 17 Schematic representation of the suggested difference between the effective and actual reduction in the surface area of the C-18 silica available for desolvation of solutes by the adsorbed pairing ion;

(a) Area available for desolvation is (As - eA);

(b) Assumed surface area available for desolvation is (As - aA).

Method of determination	Column void volume in cm^3
Maximum void volume (Karl Fischer)	2.58
Sodium nitrate retention	2.45
Potassium bromide retention	2.40
Potassium phosphate retention	2.16
Potassium dichromate	1.92

Table 1

Comparison of the values of the void volume of a column (25 x 5 mm), obtained by different methods.

Solute	Pairing ion	K_6	$K_6^{\frac{1}{2}}$	K_1	$K_1^{\frac{1}{2}}$
NA	TEA	0.223		-0.778	
2,4DMBA	TBA		0.0051		2.89
AN	CAMS	0.031		-0.612	

Table 2

$K_6^{1/n}$ and $K_1^{1/n}$ values of Equation (28) calculated to produce the best fit of experimental data.

(A)

Solute	$K_{des} \times 10^8$	$K'_{des}K_{IE}^{\frac{1}{2}}(TMA) \times 10^5$	$K'_{des}K_{IE}(TEA)$	$K'_{des}K_{IE}^{\frac{1}{2}}(TBA) \times 10^4$	$K'_{des}K_{IE}^{\frac{1}{2}}(CETA)$
BA	2.28	2.18	0.103	1.69	0.104
2,4DMBA	9.52	11.48	0.519	5.76	0.208
NA	15.7	18.76	1.05	16.5	
4EBA	24.4	26.23	1.36	17.9	

(B)

Solute	$K_{des} \times 10^8$	$K'_{des}K_{IE} \times 10^2$			
		PANS	CAMS	OCTS	SLS
AD	0.711	3.757	2.827	1.194	1.293
AN	1.41	15.12	7.295	8.718	20.153
DOP	1.99	11.49	7.484	3.825	4.405
BZ	3.52	34.093	16.656	17.429	27.35

Table 3

K_{des} and $K'_{des}K_{IE}$ values calculated to produce the best fir of experimental data (A) cationic pairing ions with acidic solutes, (B) anionic pairing ions with basic solutes.

(A)

Solute	$K'_{IE} \times 10^{-6}$			
	TMA	TEA	TBA	CETA
BA	0.922	4.54	55.4	4.50
2,4DMBA	1.45	5.45	36.3	2.60
NA	1.41	6.67	100.9	
4EBA	1.15	5.58	53.8	

(B)

Solute	$K'_{IE} \times 10^{-6}$			
	PANS	CAMS	OCTS	SLS
AD	5.284	3.976	1.679	1.819
AN	10.723	5.174	6.183	14.56
DOP	5.774	3.761	1.922	2.214
BZ	9.685	4.732	4.732	7.77

Table 4

K'_{IE} values calculated for solute-pairing systems assuming K'_{des}/K_{des} is constant; (A) cationic pairing ions with acidic solutes and (B) anionic pairing ions with basic solutes.

Solute	$K'_{des}K_{IE} \times 10^2$ (SLS)		Solute	$K'_{des}K_{IE} \times 10^2$ (CETA)	
	30% v/v Acetonitrile	60% v/v Acetonitrile		30% v/v Acetonitrile	60% v/v Acetonitrile
AD	0.772	0.614	BA	1.228	0.748
AN	2.932	1.521	2,4DMA	2.376	1.026
DOP	1.087	0.702	NA	4.139	1.270
BZ	4.582	1.947	4EBA	4.876	1.415

Table 5

$K'_{des}K_{IE}$ values calculated to produce the best fit of experimental data in mixed organic/aqueous solvent.

CHAPTER THREE

A STUDY OF THE DECOMPOSITION OF D-GLUCOSE DURING AUTOCLAVING AND IDENTIFICATION OF ACIDS PRODUCED USING REVERSED PHASE ION-PAIRING HPLC

3.1 Introduction

D-glucose solutions are known to undergo changes when heated; the most important being a lowering of pH and the development of a yellow colour [156-160]. Various suggestions have been made as to the source of the yellow colour and several different acids have been suggested as being present after autoclaving [161-166].

Despite the abundance of literature on this reaction the natures of the coloured material and the acidic products as well as the mechanism of their formation remain uncertain. It has been stated by Fetzer et al. that the literature on this process is extensive and confusing [167].

However, it has been well established that heating D-glucose and other hexoses in acidic or neutral aqueous solution leads to the production of 5-hydroxymethylfurfuraldehyde (5HMF) [168-170]. The rate of formation of 5HMF has since been taken as an indication of the amount of D-glucose decomposed [162,163,166,171,172].

When D-glucose is refluxed in aqueous acid under open air conditions, 5HMF is not the final product and further reaction results in the formation of levulinic and formic acids [173]. This very early work has been generally taken to account for the acidity produced under autoclaving conditions. It has been indicated by Joslyn [174] that compounds with both hydroxyl and aldehyde groups can polymerize to yield brown pigments. The presence of these reactive aldehyde and hydroxyl groups in 5HMF suggests that this compound may polymerize. The degree of colouration in thermally degraded D-glucose solutions has been stated to be at least partly due to the polymerization of 5HMF [170] and it has been shown to parallel the extent of the formation of 5HMF from D-glucose [171].

On the other hand, the work by Wolform et al.[161] in the study of the conversion of D-glucose to 5HMF by spectrophotometric methods has suggested that some intermediates are formed prior to the production of

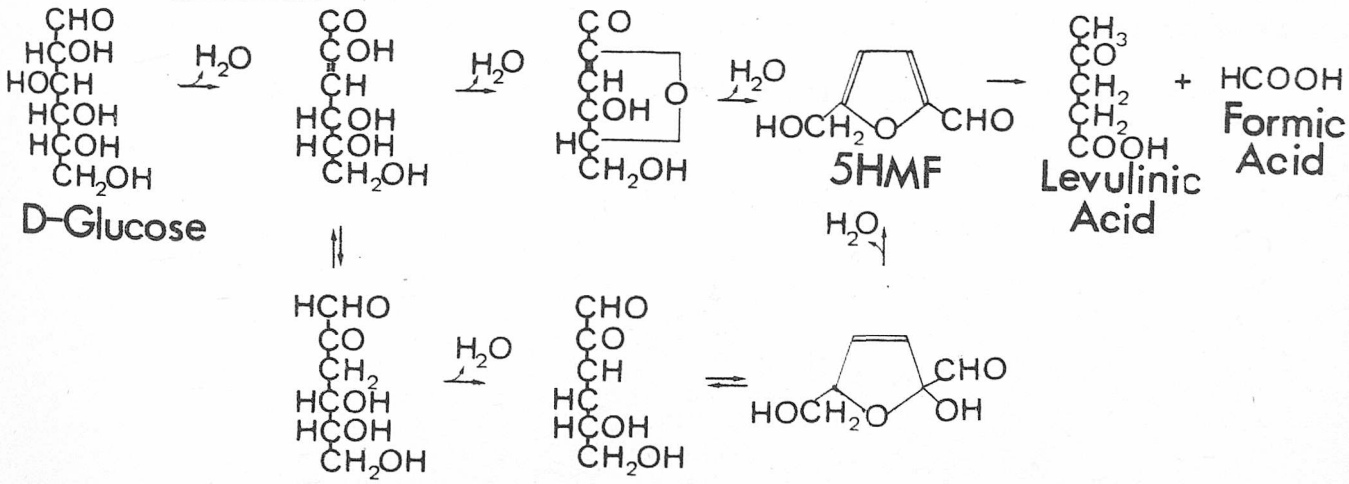
5HMF. On the basis of this, structures of various ketohexoses have been proposed. Fleming et al.[175] have also suggested that polymerization of such intermediates is the cause of the colour in thermally degraded D-glucose solutions.

Although numerous hypotheses have so far been advanced for the decomposition of D-glucose on heating, only four clearly distinguishable mechanisms have appeared in the literature. These mechanisms will be discussed in detail below.

3.1.1 Mechanism proposed by Wolform et al.[161]

This mechanism is by far the most widely accepted for the decomposition of D-glucose solution on heating. A quantitative spectrophotometric study showed that 5HMF is characterised by a major adsorption band at 285 nm and a minor band at 228 nm. However, studies of the UV spectrum of degraded D-glucose solutions as a function of heating time showed that the band at 228 nm is far more pronounced than that at 285 nm in the early stages of D-glucose decomposition. Only after prolonged heating, more than four hours, will the UV spectrum of the solution resemble that of 5HMF. On the basis of this finding, it was suggested that the band at 228 nm is not due to the production of 5HMF at the early stages of decomposition but due to the formation of intermediates from the D-glucose. On this evidence a reaction mechanism was proposed. This is shown in Scheme (4) which indicates the proposed structures of the intermediates.

Scheme (4)



This suggestion received further support from the finding that the rate of formation of 5HMF is not proportional to the concentration of D-glucose present [172]. The rate constant of the degradation of D-glucose calculated from measurements of 5HMF was also found to be higher than that calculated from D-glucose depletion [162]. This was taken to indicate that at least one compound other than 5HMF was being produced and measured. The presence of at least one other substance in the early stages in the degradation process has been confirmed by Wing using Folin-Ciocalteau reagent [176].

Whilst the general scheme of D-glucose decomposition has widely been accepted, the structures of the intermediates are still uncertain and different structures of these intermediates have been postulated [175,177].

3.1.2 Mechanism proposed by Taylor et al. [163]

Most of the experiments mentioned above were carried out under open air conditions, which were not appropriate to any decomposition produced during autoclaving. Using spectrophotometric methods, it was demonstrated that the formation of 5HMF from D-glucose in a sealed container exhibits an induction period. This was interpreted as due to the formation of intermediate compounds. However, it was also noticed that the decrease of the pH of the D-glucose solution occurred at the very early stage of the decomposition when a negligible amount of 5HMF had been produced. Furthermore, on heating 5HMF solution under the same conditions very small rate constants were measured for the decomposition of 5HMF. It was thus suggested that the pH decrease was not produced by the decomposed products of 5HMF but by an intermediate. Accordingly a reaction mechanism was proposed and is represented in Scheme (5).

Scheme (5)

D-glucose ⟶ Intermediate(s) + H^+

Intermediate(s) $\xrightarrow{H^+}$ 5HMF ⟶ Products

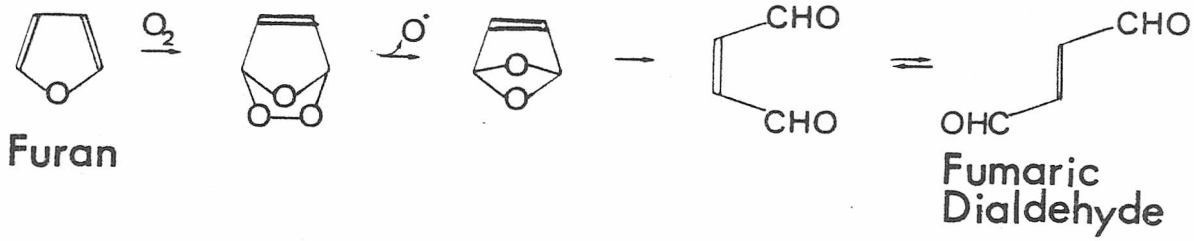
In this study the acidic intermediate(s) and the products were not specified.

3.1.3 Mechanism proposed by Tahir and Cates [164]

In this work the authors suggested that the early acidity is related to the appearance of a product absorbing radiation at a wavelength of 228 nm rather than to an intermediate as indicated above.

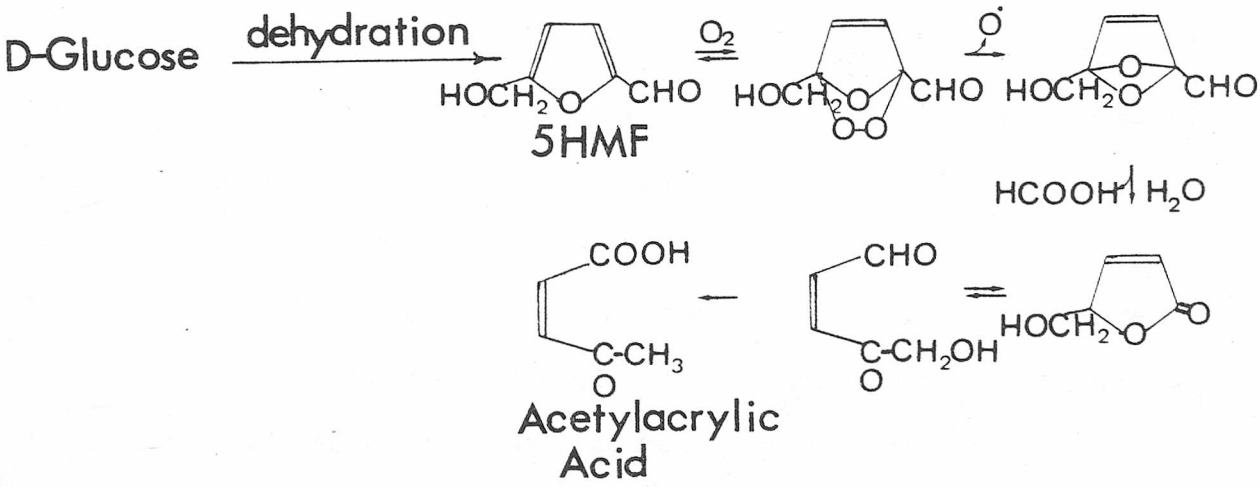
Based on the Schenk oxidation mechanism as shown below in Scheme (6),

Scheme (6)



and on the fact that fumaric dialdehyde has a spectrum showing a major band at 227 nm, it was postulated that the 5HMF found in the early stages will oxidise to form intermediates and acetylacrylic acid as shown in Scheme (7).

Scheme (7)



The above chemical reactions have been assumed to occur at the early stages of D-glucose decomposition. At the later stages, the oxidation of 5HMF slows down due to an auto-inhibition process [179] and hydrolysis of the 5HMF will take place with the formation of levulinic and formic acids as previously suggested.

Since acetylacrylic acid gives a yellow colour when dissolved in water, has a maximum in UV adsorption at 228 nm and is acidic in nature it would seem to be the best candidate to explain all the common phenomena that are observed in degraded D-glucose solution.

The formation of this acid from 5HMF, however, requires a very rigorous oxidation process which may not correspond to the rather mild autoclaving condition in sterilising D-glucose infusions. There is very little direct experimental evidence to confirm the presence of acetylacrylic acid in such solutions.

3.1.4 Formation of metasaccharinic acid at the early stages of D-glucose decompositoin [165]

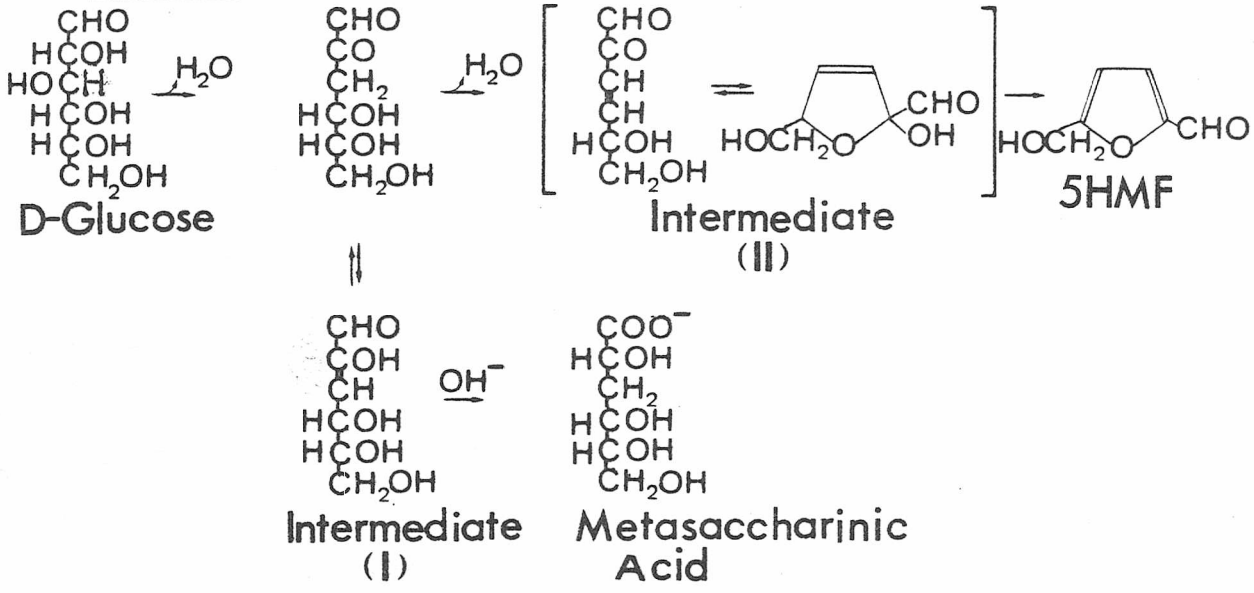
The basis of all measurements that have hitherto been discussed are spectrophotometric. The use of HPLC to study of initial stages of D-glucose degradation was first performed by Taylor and Sood [165].

In their study, Partisil ODS was used as stationary phase with water as solvent and wavelength of detection at 254 nm. On the basis of the time dependence of chromatographic peaks on heating, in addition to the main product 5HMF, two intermediates and an acidic product were detected. By use of ion-pair techniques with tetrabutylammonium as pairing ion, the retention time of the acid was markedly increased. The sequence of formation of all the compounds was interpreted as follows. An intermediate was first produced followed by the appearance of the acid, subsequently another intermediate was detected before the formation of

5HMF. Based on these observations, it was postulated that the acid was produced from an intermediate in the early stages of the D-glucose decomposition. As the pH of the solution decreases due to the formation of acid, the equilibrium shifts to favour the formation of a second intermediate which further decomposes to produce 5HMF.

The sequence of reactions is represented by Scheme (8).

Scheme (8)



This suggestion is supported by the fact, that while acid conditions of heating D-glucose forms 5HMF [161,162], it is also well established that under basic conditions intermediate (I) in Scheme (8) has been identified during the hydrolysis of D-glucose solutions [182]. There is however little evidence as to the nature of the acid formed other than the time at which the acid appears during the reaction sequence. Also the detection of such a non UV absorbing as metasaccharinic would be very insensitive at 254 nm and would require high concentrations to be present.

As well as levulinic and formic [173], acetylacrylic [164] and metasaccharinic acids [165] which have already been mentioned, the literature contains many more references to acidic products which are derived from D-glucose. Other acids as gluconic [183], pyruvic and lactic acids [182]

have also been suggested as the by-products of D-glucose decomposition and may contribute at least to some extent to the acidity of the solution.

The direct formation of organic acids usually involves strongly oxidising and extremely acidic or basic conditions. Such reactions result in the loss of one or more carbon atoms to produce aldonic and other acids [185]. Such drastic conditions do not parallel cycles used for the autoclaving of D-glucose in neutral solution.

Enormous problems have to be faced in the investigation of the intermediates and acids produced during autoclaving of D-glucose. A major difficulty lies in the relatively small proportion of the sugar decomposed and the subsequent low concentration of impurities produced when heated under mild conditions in sealed containers. Isolation and characterisation of the products other than 5HMF are extremely difficult especially in the presence of a comparatively large excess of glucose.

3.2 Scope of investigation

It would appear from the above that the decomposition of D-glucose is very complex and that considerable confusion exists in relating reactions occurring under widely different experimental conditions. The impurities arising from D-glucose decomposition during autoclaving are controlled by the various pharmacopoeias. It would therefore be of considerable interest if further information can be obtained regarding the intermediates and products formed during such mild decomposition conditions. This is relevant since control of impurities is based mainly on the assumption that 5HMF is the only UV absorbing species present. Indeed 5HMF concentration has been extensively taken as a measure of D-glucose decomposition.

Until recently, with the advent of HPLC techniques, no specific analytical method was available for the study of such complex reactions

especially those involving thermolabile materials such as sugars. HPLC, especially in conjunction with ion-pairing techniques would appear to offer a useful method of establishing the components of such a degradation. The major limitation is, however, the inadequacy of detection. This is limited, at low concentration to those compounds having appreciable adsorption in the ultraviolet.

In view of the information contained in Chapter (2), it would seem appropriate to re-examine the D-glucose decomposition reaction under autoclaving conditions since a much greater control of the chromatography of both neutral and ionised solutes is now possible.

It was intended to retain the acidic products that are produced in the early stages of D-glucose decomposition upon heating by the use of ion-pair chromatography. The retention behaviour of the acidic products would then be compared with that of the previously suggested acids. The UV adsorption spectra of the acidic products from the decomposed D-glucose solution were also intended to be recorded by the use of the stop flow scanning technique. It was hoped that such chromatographic and spectral data when compared with similar data for standard acids would allow unequivocal identification of acidic products formed during the D-glucose degradation.

It was also the aim of this present investigation to study the mechanism of degradation of the D-glucose solution under autoclaving conditions as well as examining the browning reaction that is usually observed on prolonged autoclaving.

It has been reported by Sing and other [171] that the decomposition of the D-glucose solution in terms of 5HMF production is pH dependent and the solution is most stable at pH 3. It was thus envisaged that the formation of the intermediates and the acidic products could also be pH dependent. The effect of pH on the D-glucose decomposition was therefore also investigated.

5HMF is generally believed to be the source of the acids and the cause of the brown colour of the degraded D-glucose solution. On the other hand it has been reported that 5HMF solution is very stable on heating [163]. It was also intended in this study to examine these contentions using HPLC.

3.3. Experimental

Apparatus

Chromatography was carried out using a variety of equipment comprising Applied Chromatography System (750/3) and Waters Associates (M6000A) constant flow pumps. Waters (M440) (254 nm) and Pye-Unicam (LC3) variable ultraviolet detectors were used. Chromatographic columns used ranged from 70-200 mm depending upon the resolution and retention times required and columns were 4.6 mm internal diameter. Columns were slurry packed with ODS-Hypersil 5 μm (Shandon, London, Great Britain) as previously described in Section (2.3.3). Injection was by a Rheodyne 7125 valve fitted with 20 or 100 μl loops. Stopped flow measurements were made in a Pye-Unicam (SP1800) and Cecil (CE588) spectrophotometers adapted to take a 10 μl flow cell. pH measurements were made using a Pye-Unicam 290H meter with a combined micro glass/silver chloride electrode system.

Materials

Pairing ions, tetraethylammonium (TEA) and tetrabutylammonium (TBA) as their bromide salts were supplied by Aldrich Chemical (Milwaukee, WI, U.S.A.), D-glucose was obtained from May and Baker Ltd. (Essex, Great Britain) and samples of D-glucose solutions having been autoclaved by an accepted regimen were obtained from Aberdeen Royal Infirmary. Buffer salt, disodium hydrogen phosphate, was purchased from Fisons Scientific Co. (Leicester, Great Britain). Water used was double distilled and Millipore filtered.

Methods

Several types of measurements were made in this investigation. HPLC chromatographic data were recorded on the reaction mixture as a function of time. D-glucose 10% w/v solution in distilled water was heated in sealed 1 cm^3 glass ampoules in a air oven at 120 ^{o}C. At suitable intervals i.e. every 15 to 30 minutes, several ampoules were removed and quickly plunged into ice to cool the contents rapidly. 20 µl of the contents were directly injected for chromatography.

Decomposition of 5HMF was followed in the same way as described above at a concentration of 0.027% w/v, which is equivalent to the concentration of 5HMF produced on heating 10% w/v D-glucose solution for 8 hours.

Isolation of the intermediates from the degraded D-glucose sample was achieved by collecting fractions of the sample at the outlet of the detector during chromatography. The samples were then concentrated by freeze drying.

The isolation of the stable acidic products was achieved using a similar procedure but with a very concentrated sample injected for chromatography. The concentrated sample was prepared by autoclaving 500 ml of D-glucose (10% w/v) for several hours at 120^{o}C. A syrup was prepared from this solution by evaporating the water under vaccum. The pH of the syrup was then adjusted to pH 1 by addition of hydrochloric acid, followed by shaking vigorously with 50 ml of diethylether in a separating funnel. The aqueous syrup was then transferred to another funnel and the above procedure was repeated with a further addition of 50 ml of diethylether. The ether extract which contained the acidic products and many other non-ionic compounds e.g. 5HMF and the brown pigments, was allowed to evaporate to dryness in a fume cupboard. The

residue was then dissolved in a minimum of water and subjected for chromatographic isolation of the acidic products.

pH measurements of the solutions were carried out directly by the use of a pH meter coupled with a microelectrode prior to chromatography.

3.4 Results and discussion

The complexity of the degradation pattern of D-glucose can be demonstrated by the chromatogram of the D-glucose solution after heating for one hour at 120°C, as shown in Figure (18), with wavelength of detection at 254 nm. However, when the chromatograms of the same samples were recorded at several wavelengths ranging from 220 nm to 300 nm one additional peak was detected at high wavelengths. These results are represented in Figure (19).

Figures (18) and (19) demonstrate that there are several ultraviolet absorbing species present in the degraded D-glucose solution beside the well known product 5HMF. Figure (20) shows the variation of peak height of all compounds detected as a function of time of heating and also shows the variation in pH of the reaction mixture. The essentially constant D-glucose concentration was detected as a marked refractive index peak in all chromatograms using the LC-3 detector.

A and B were designated as acidic products due to their elution at or before the solvent front in water together with the marked increase in retention times on addition of low concentrations of quarternary ammonium pairing ions (TEA and TBA). Representative chromatograms under ion-pairing conditions are illustrated in Figures (21a) and (21b).

The acidic natures of A and B were further demonstrated by the technique of ion-suppression. Figure (22) shows the variation of their capacity factors as a function of pH. It can be observed that the acids are strongly retained at low pH, where they exist in the undissociated forms. At high pH, the fully ionised acids are rapidly eluted. From

Figure (22), the pKa values of both acids were evaluated as approximately 3 [73,124].

Furthermore, as illustrated in Figure (20), the pH value of the degraded D-glucose solutions as a function of heating time decreases as the concentration of A and B increase.

Peaks 3, 4 and 5 were regarded as intermediates because the concentration of these substances, when expressed in terms of peak height, exhibited initial rises followed by decreases after reaching maxima. Using the stopped flow scanning technique, the UV spectra of intermediates 3, 4 and 5 were recorded and are shown in Figure (23). Intermediate 3 was found to exhibit a maximum adsorption at 235 nm, whilst 4 and 5 were shown to have maxima at 285 and 230 nm respectively. These results, however, make little contribution to the understanding of the structures of these intermediate compounds.

3.4.1 Sources of the acidic products and of the coloured substances on heating D-glucose solution

Heating aqueous solution of 5HMF (0.027% w/v) for 8 hours at 120°C with subsequent chromatography in aqueous buffer mobile phase [185] and ion-pairing solvents showed no decomposition of this product. No evidence of acids A and B was observed. The pH did not change nor was any colouration apparent in the solution. However, isolation of peaks 3, 4 and 5 individually, followed by heating of their solutions and subsequent chromatography with an ion-pairing solvent produced peaks corresponding to A, B and 5HMF as shown in Figure (24). The broad peak proceeding those of A and B could not be identified. Also the acids and 5HMF present were found to be in a much higher concentration than usually detected in the D-glucose solution. Such findings besides confirming the intermediate natures of 3, 4 and 5 may also lead to the suggestion that the acidic

products A and B are derived by decomposition of the intermediates. It was also observed that the solution became dark in colour, which supports the contention that an α-diketohexose intermediate itself polymerises to formed coloured material [175]. It can also explain the observation that autoclaved D-glucose solutions may sometimes exhibit brown colouration while containing negligible concentration of 5HMF. On the other hand it has been observed that degraded samples of D-glucose solution left for some time at room temperature after brief exposure to air showed an increase in acidic products at the expense of 5HMF [186]. Such observations cast doubt on the idea that the acids A and B were derived from the intermediates. Accordingly it may be that A and B were produced from the 5HMF by some mild oxidation reaction, possibly catalysed by the presence of D-glucose or intermediates.

To distinguish between the above two possible sources of the acidic compounds A and B it was necessary to attempt identification of these compounds.

3.4.2 Identification of acids A and B

The isolation of sufficient quantities of acids A and B was not possible due to their low concentrations. Attempts to characterise these acids therefore concentrated on comparison of their chromatographic behaviour with standard acids. In Chapter (2) the k' value of acids was shown to depend in a characteristic fashion on pairing ion concentration. It was considered that this might provide a sensitive test for identity by comparing with standards particularly if different pairing ions were used.

Figures (25) and (26) show the behaviour of acids A and B together with several previously suggested D-glucose produced acids in TEA and TBA as pairing ion respectively.

Sufficiently high concentrations of the low molar absorptivity acids such as levulinic, formic and gluconic were used to ensure their detection. It is obvious from these figures that neither A nor B correspond to any of the acids previously suggested. The behaviour of acid A in TEA closely corresponds to that of acetylacrylic but in TBA their behaviours are markedly different.

The value of such a process in this identification is enhanced by the fact that the elution order of acids A and B are reversed in the two different pairing ions used at concentrations above 2 mM in TBA. The elution order of standard acids are also reversed in some cases e.g. pyruvic and lactic. Such a change in selectivity as a result of using different pairing ions and pairing ion concentration has been attributed to the change of the bound counter ion concentration in the electrical double layer formed on the C-18 surface when different pairing ions are used [153].

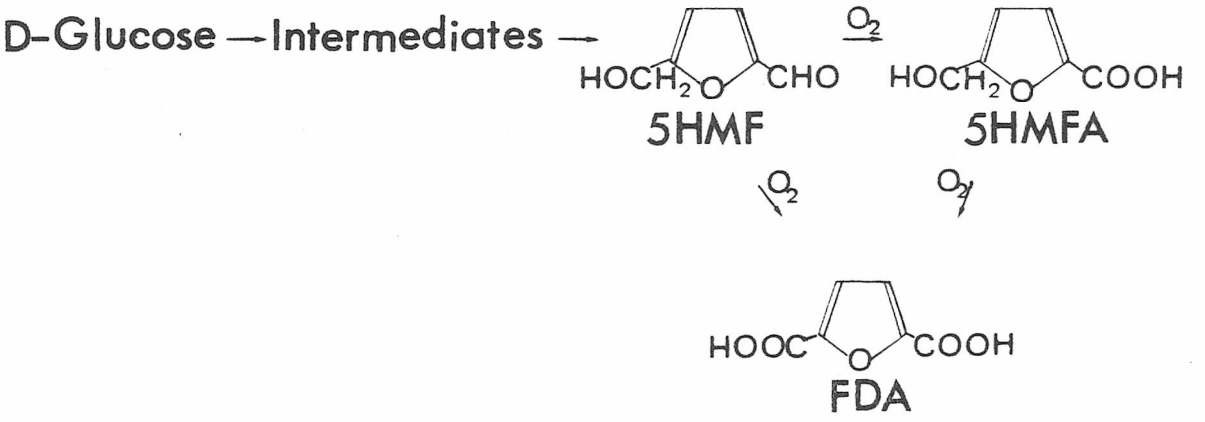
These results obtained chromatographically are consistent with the ultraviolet spectra of A and B obtained by the stopped flow technique and shown in Figure (27). Both of these acids show maxima at considerably higher wavelengths than would be expected for any of the previously suggested acid products. It was thus considered most probable that A and B are acids produced by further reaction possibly by oxidation 5HMF.

Samples of two acids, 5-hydroxymethylfuroic acid (5HMFA) and furan-2,5-dicarboxylic (FDA) were made available. These had been synthesised by direct oxidation of 5HMF using silver oxide [187]. The chromatographic behaviour of these acids is also shown in Figures (25) and (26) and their ultraviolet spectra in Figure (27). In both of these comparisons acid A is seen to be identical with 5HMFA and acid B with FDA. It would appear from this that the acids observed in the present work arise due to the oxidation of 5HMF during the heating process. This may explain

by relatively low concentration of A and B are normally obtained during the autoclaving process; the reaction being limited in terms of oxidation in the sealed system.

This may also explain the apparent stability of 5HMF when measured in a closed system. It does not, however, support the idea that the acids are obtained directly from the intermediates and the experimental results indicating this must be re-interpreted on the basis that in isolating the intermediates additional oxygen is available so that once 5HMF is formed additional quantities of this may be oxidised to the acids as indicated in Scheme (9) shown below.

Scheme (9)



It is not possible to say from the above experiment that acid B arises from further oxidation of acid A or independently from 5HMF.

No standard metasaccharinic acid was available in this study, but in view of its heavily hydroxylated structure and lack of any UV absorbing functional group, it is very unlikely that it will correspond to the chromatographic behaviour or adsorption spectra of acids A or B.

3.4.3 The effect of pH on the decomposition of D-glucose upon heating

It has been demonstrated by Singh et al.[171] that the formation of 5HMF from D-glucose upon heating is minimum at pH 3. It is also interesting to notice from Figure (20) that the concentration of all intermediates 3,

4 and 5 start to decrease at pH 2&2.5. It is thus possible that the decrease in the pH of the D-glucose solutions will act to reduce the rate of formation of intermediates from D-glucose resulting in a decrease in the rate of formation of 5HMF.

In order to examine the above hypothesis D-glucose solutions (10% w/v) were initially adjusted to pH 2, 2.5, 3 and 4 respectively by the addition of dilute hydrochloric acid. The presence of chloride ion has been shown to have minimal effect in D-glucose decomposition [188]. The pH adjusted D-glucose solutions were then sealed in 1 cm^3 glass ampoules and degraded by the same procedure as described in Section (3.3). The concentration of the intermediates 3, 4 and 5, 5HMF and the acidic products A (5HMFA) and B (FDA) produced at different time intervals were measured. Their concentrations in terms of arbitrary peak heights were plotted against the time of heating and compared with the results obtained from heating D-glucose solutions in water.

However, on heating D-glucose solutions which had been initially adjusted to pH 2.5 and pH 2, the typical chromatogram as illustrated in Figure (18) was not observed. The degradation process of the D-glucose appeared to be altered by such a low pH values and the complexity of the decomposition process at pH 2.5 and pH 2 is demonstrated in Figure (28). Furthermore, the solutions were observed to turn brown in colour very quickly and black precipitates appeared on prolonged heating. When the pH of the solution was initially adjusted to pH 4 and pH 3, the typical chromatograms as shown in Figure (18) was recorded. The concentration of the intermediates and products were measured and are shown in Figures (29a), (29b) and (29c). Figure (29a) demonstrated that the formation of all intermediates from D-glucose is reduced as pH decreases. In Figure (29b) it is seen that 5HMF which is the major product of decomposition of the intermediates decreases as a consequence of decrease in the

formation of its precursor at the low pH.

The acidic products 5HMFA and FDA are the oxidation products of 5HMF and decrease when the rate of formation of 5HMF decreases at low pH values. This is shown in Figure (29c). Such findings confirm the work of Singh et al. that the formation of 5HMF from D-glucose is minimum at pH 3 and the D-glucose is most stable at this pH.

3.5 Conclusions

On heating D-glucose aqueous solution in sealed containers the D-glucose will decompose resulting in the formation of three major intermediates. The intermediates themselves will polymerise to form brown coloured substances. Due to their instability, the structures of these intermediates were not identified. 5-hydroxymethylfurfuraldehyde, which is the major decomposition product in the degraded D-glucose solution is derived from the intermediates. Two acids were detected in the autoclaved D-glucose solutions and these acids were proved to be neither levulinic, formic, acetylacrylic, gluconic nor pyruvic acids. These two acid species were identified as 5-hydroxymethylfuroic acid and furan-2,5-dicarboxylic acid.

5HMFA and FDA are believed to be the oxidation products of 5HMF. However, it is still not certain whether levulinic or formic acids will be formed on heating D-glucose solution. This is because of their low UV absorbance, so that small amounts of these acids might be present but can not be detected. In view of the rather mild decomposition conditions of autoclaving it would appear unlikely that such acids will be formed from 5HMF.

D-glucose solution is found to be most stable upon heating at pH 3 and this effect is due to the decrease in decomposition of D-glucose into the intermediates.

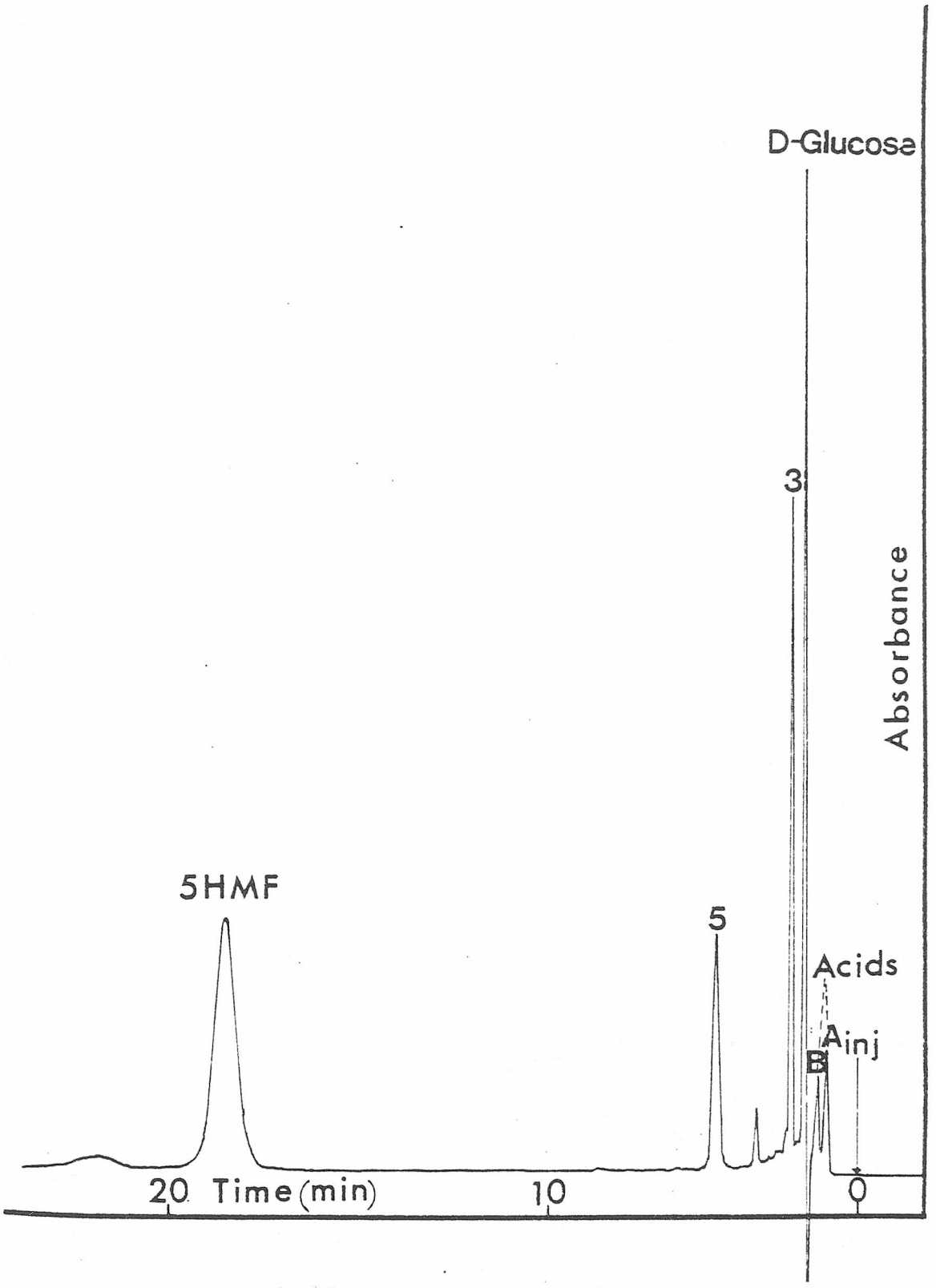


Figure 18 Specimen chromatogram of degraded D-glucose solutions, chromatographic conditions: 200 x 4.6 mm ODS-Hypersil column at 2.0 cm^3 min^{-1} flow rate; solvent-distilled water; full scale detection 0.4. Wavelength of detection 254 nm.

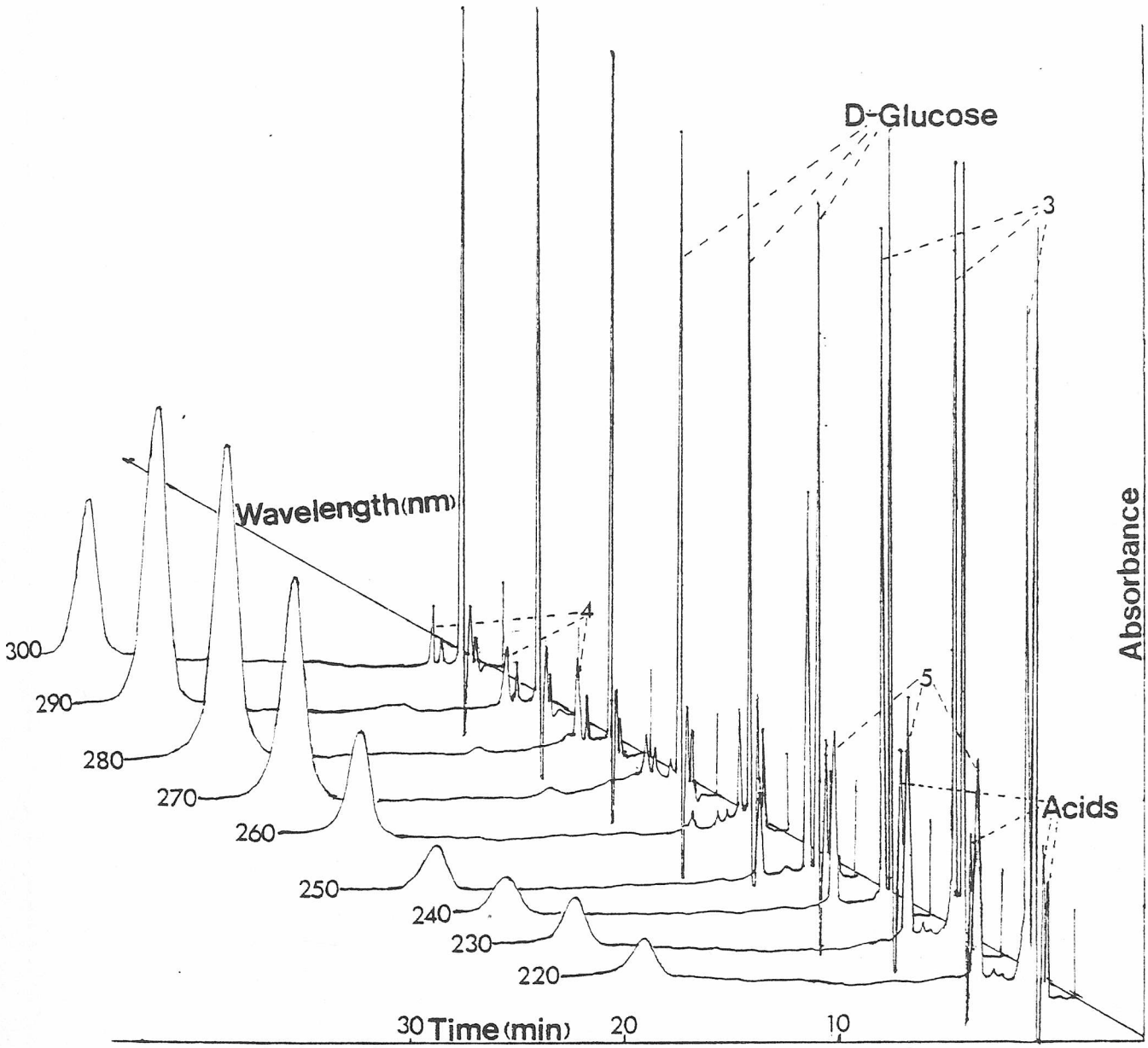


Figure 19 Graphical representation of the chromatograms of degraded D-glucose solutions at several wavelengths. Peaks referred to in text are as labelled on figure. Chromatographic conditions are as described in Figure (18).

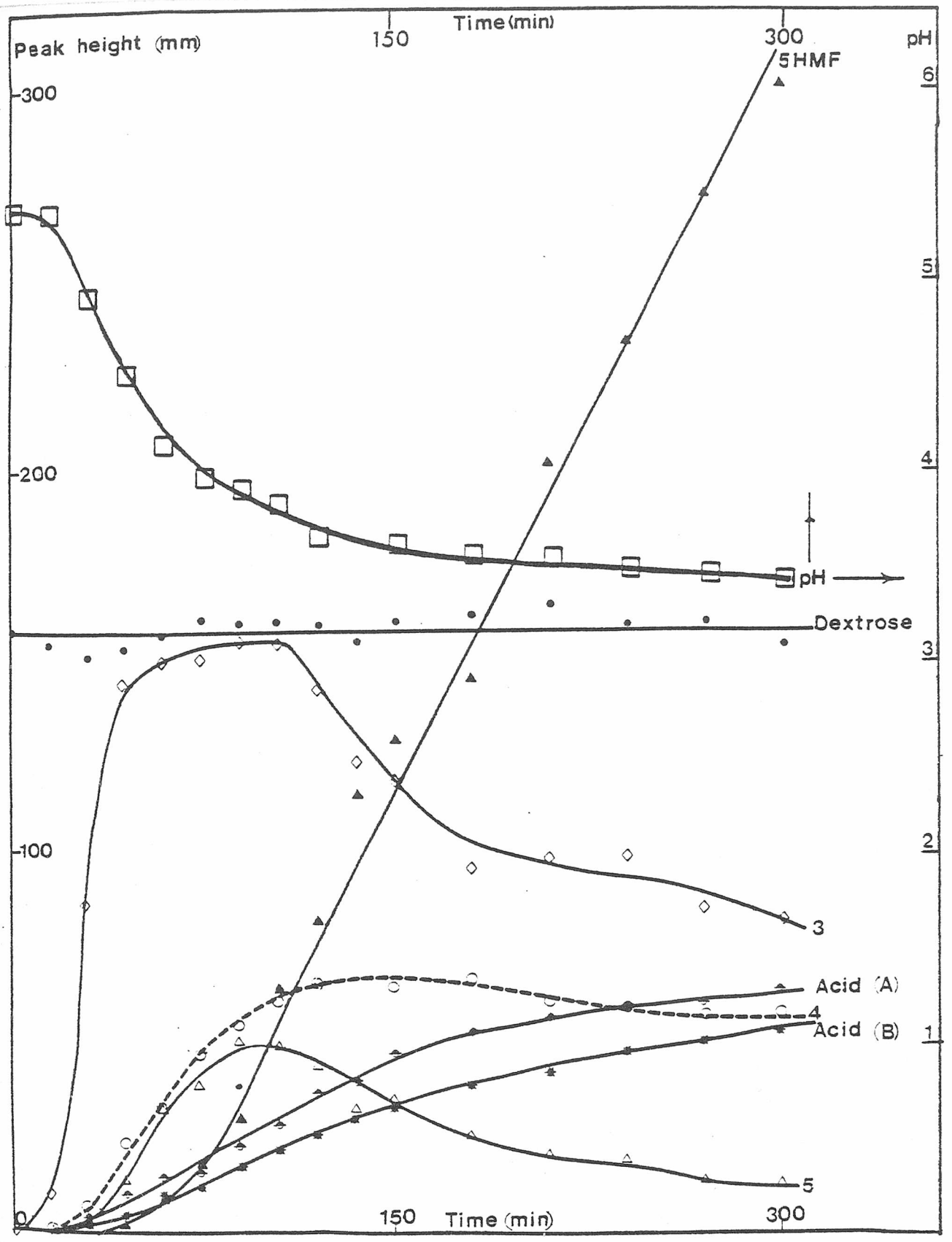


Figure 20 Variation of peak heights (corrected to full scale detection of 0.04 absorbance units) of D-glucose decomposition products with time at 120°C and also the variation in pH of the reaction mixture. Chromatographic condition as in Figure (18). Wavelength of detection of intermediate 4 was at 280 nm.

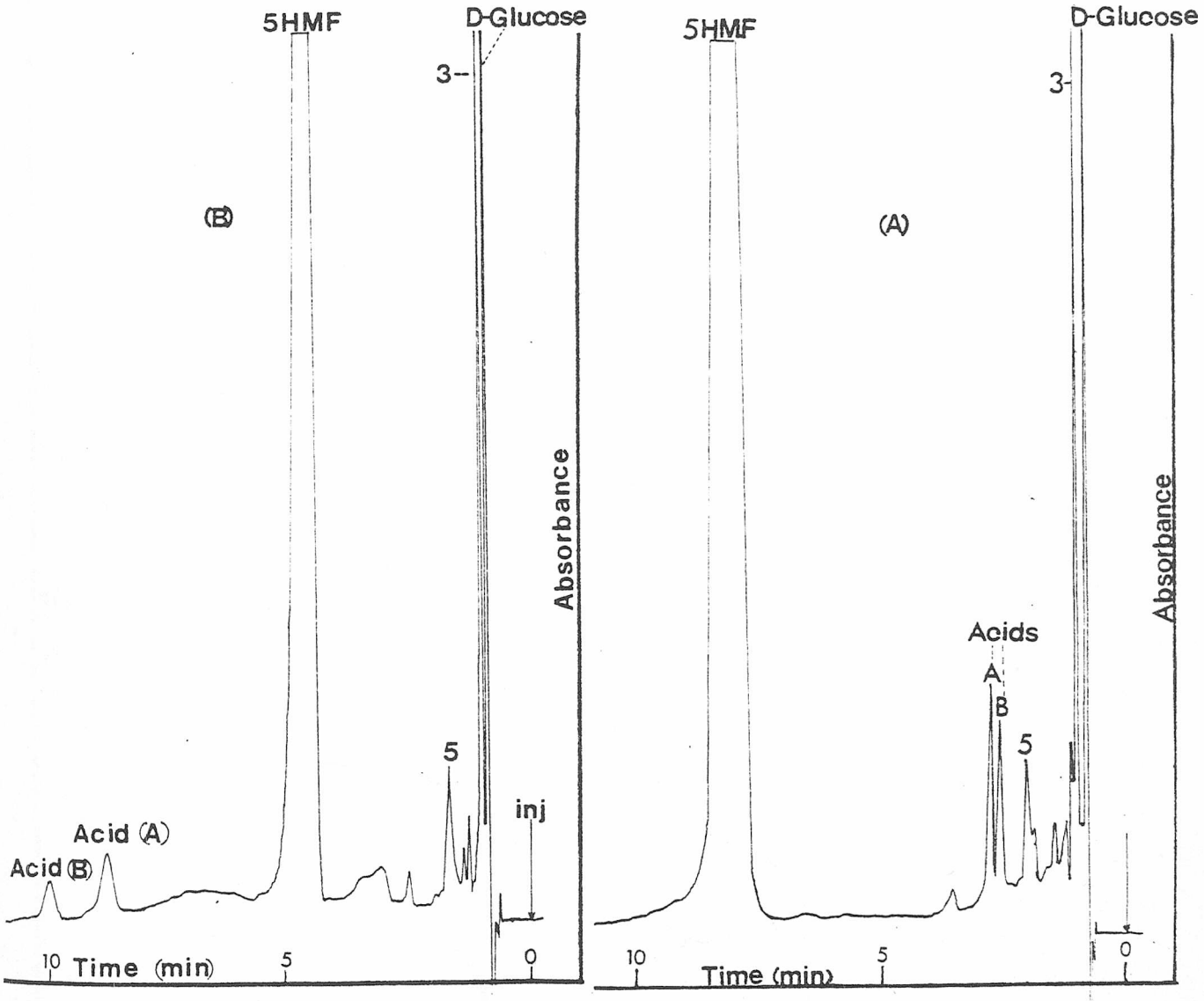


Figure 21 Specimen chromatograms of degraded D-glucose solutions under ion-pairing conditions using different pairing ions. Chromatographic conditions: 120 x 4.6 mm ODS-Hypersil column; flow rate 2.0 cm^3 mm^{-1}; solvent 5 mm disodium hydrogen phosphate buffer (pH 7) containing

(A) 20 mm tetraethylammonium bromide

(B) 5 mm tetrabutylammonium bromide.

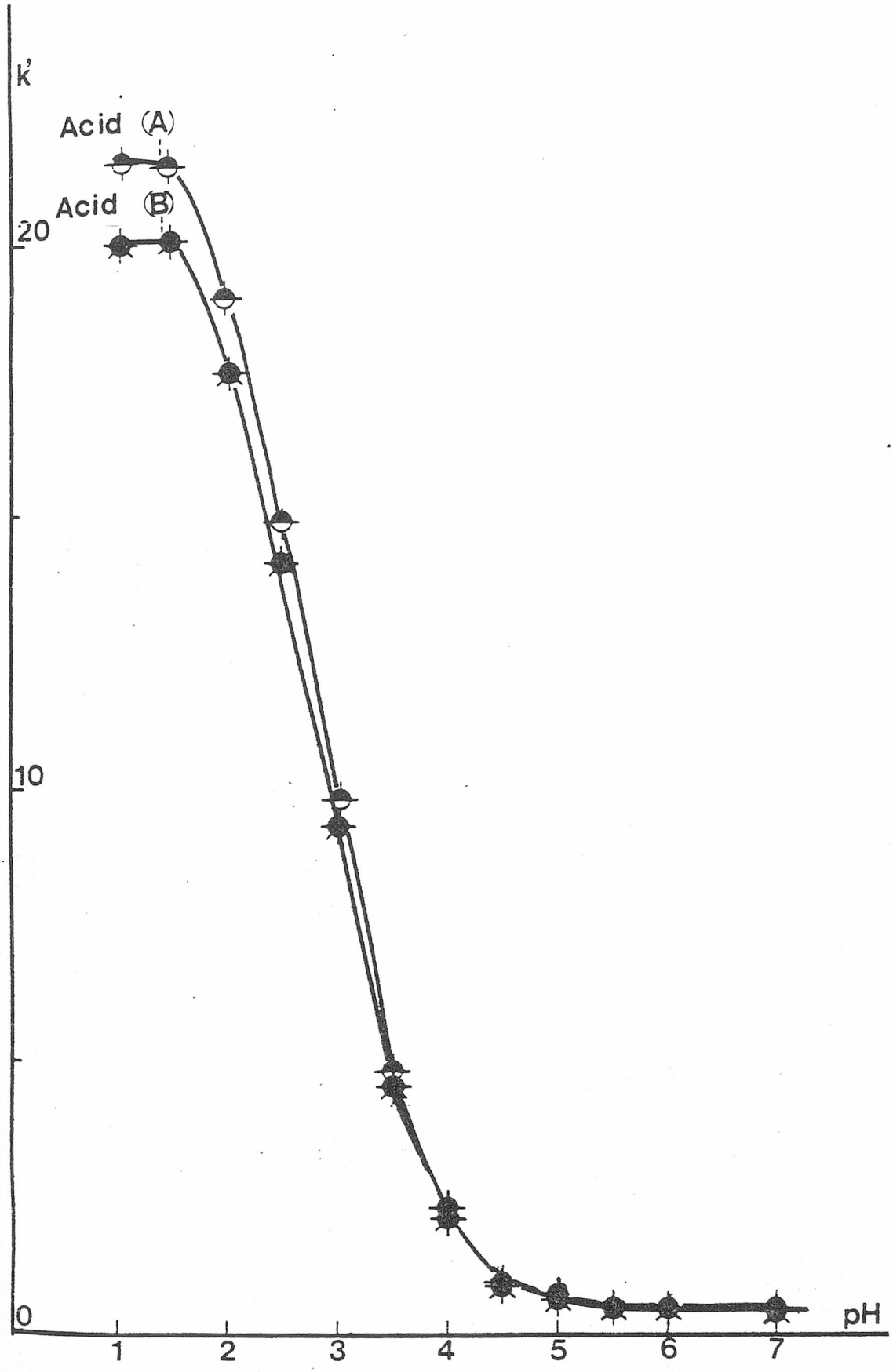


Figure 22 Plots showing the variation of capacity factors with pH for the acids A and B derived from D-glucose.

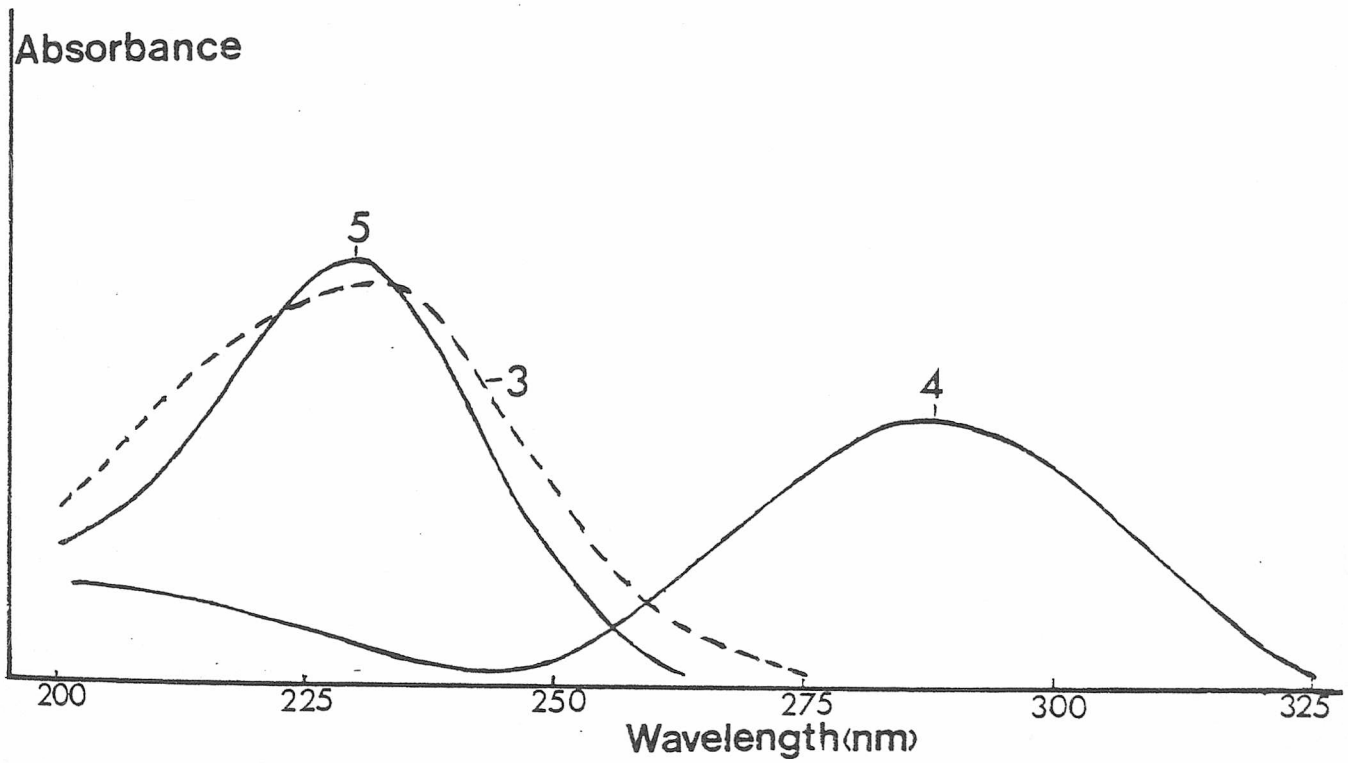


Figure 23 Ultraviolet spectra of the intermediates 3, 4 and 5 produced in degraded D-glucose solution.

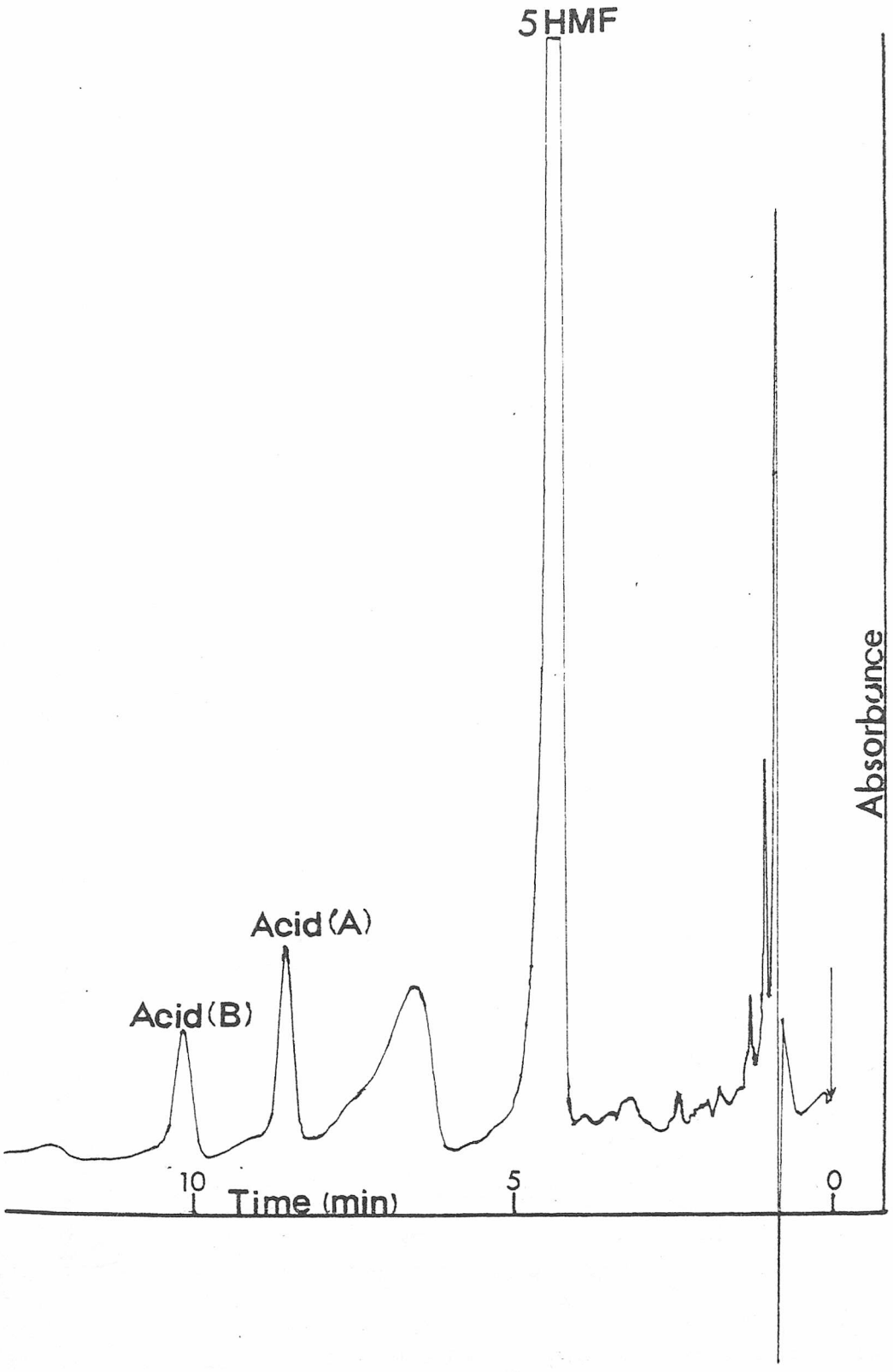


Figure 24 Specimen chromatogram after prolonged heating of solutions containing intermediates 3, 4 and 5 individually using ion-pair chromatography. Chromatographic conditions as in Figure (21B).

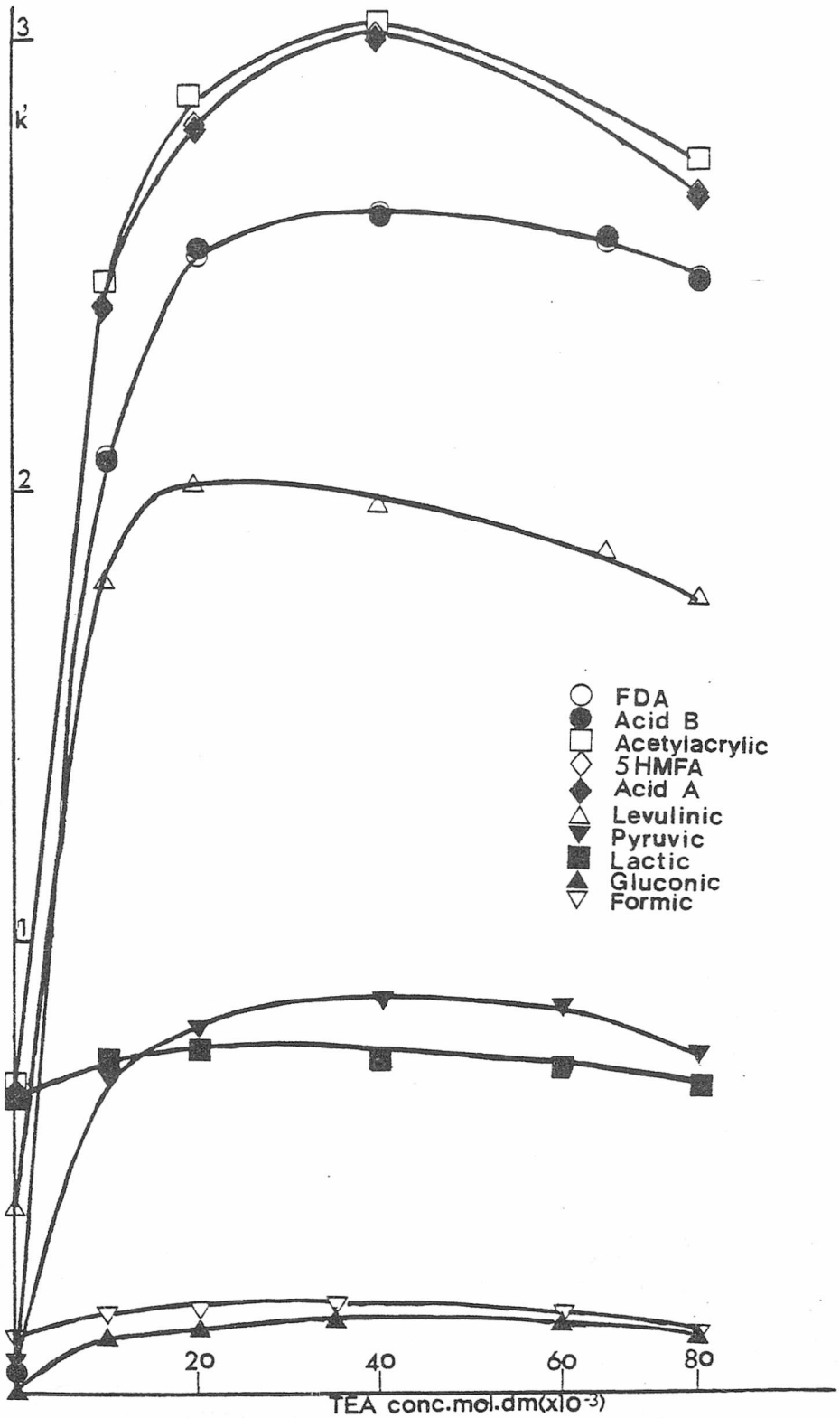


Figure 25 Plots showing the variation of capacity factor for various acids as a function of tetraethylammonium bromide concentration. Chromatographic conditions as in Figure (21A).

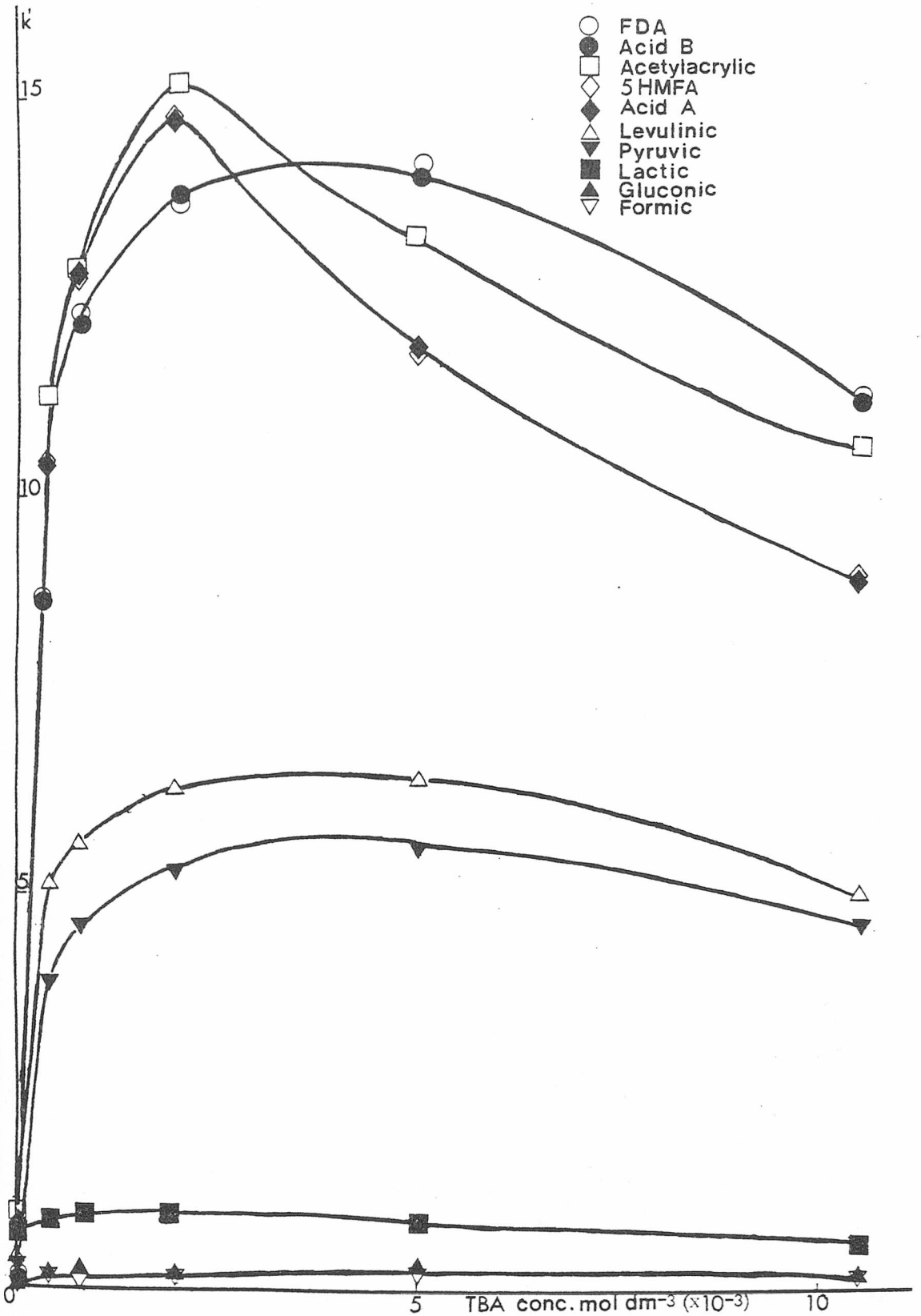


Figure 26 Plots showing the variation of capacity factor for various acids as a function of tetrabutylammonium bromide concentration. Chromatographic conditions as in Figure (21B).

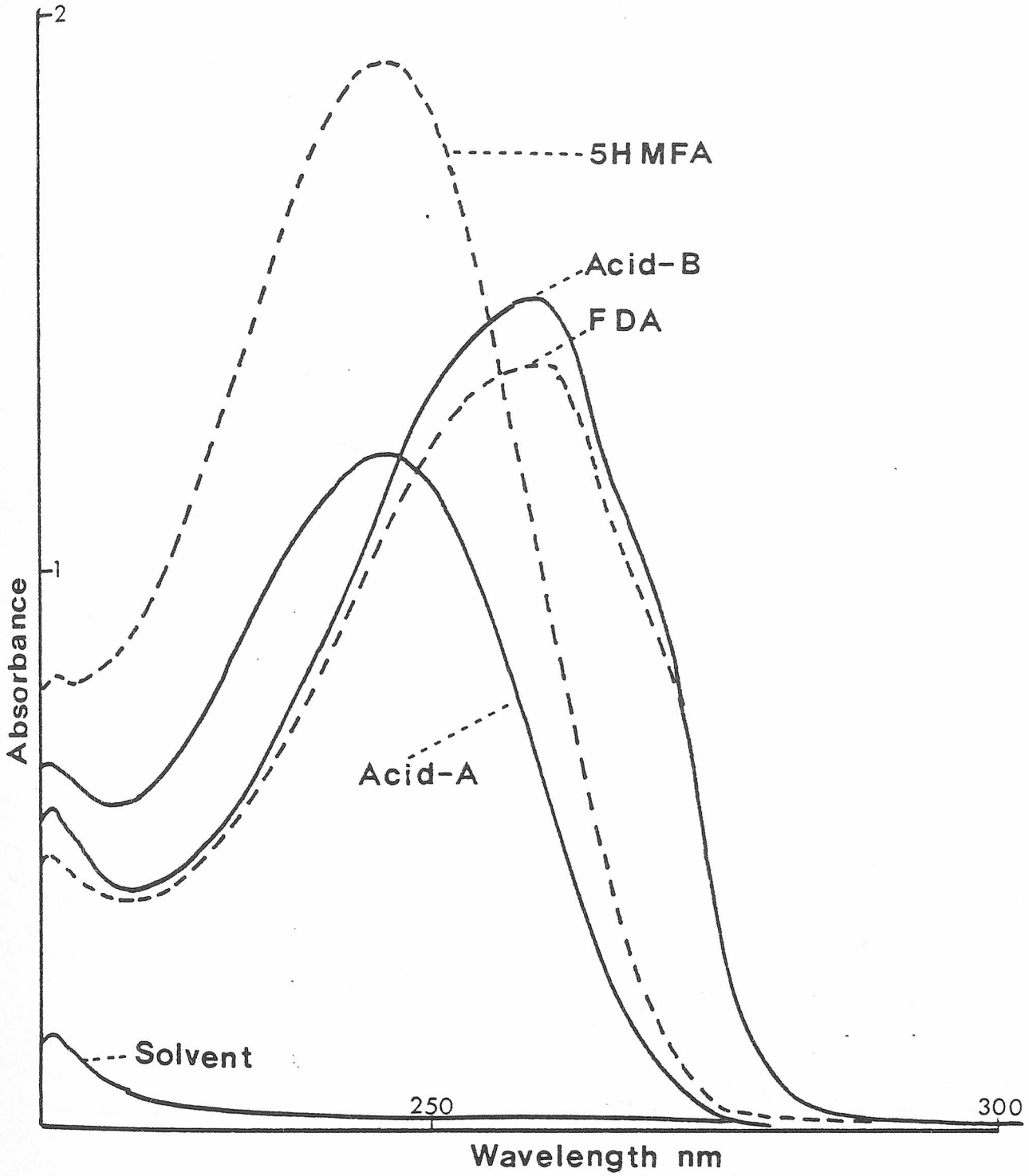


Figure 27 Ultraviolet spectra of the acids A and B produced in degraded D-glucose solution compared with standard 5HMFA and FDA.

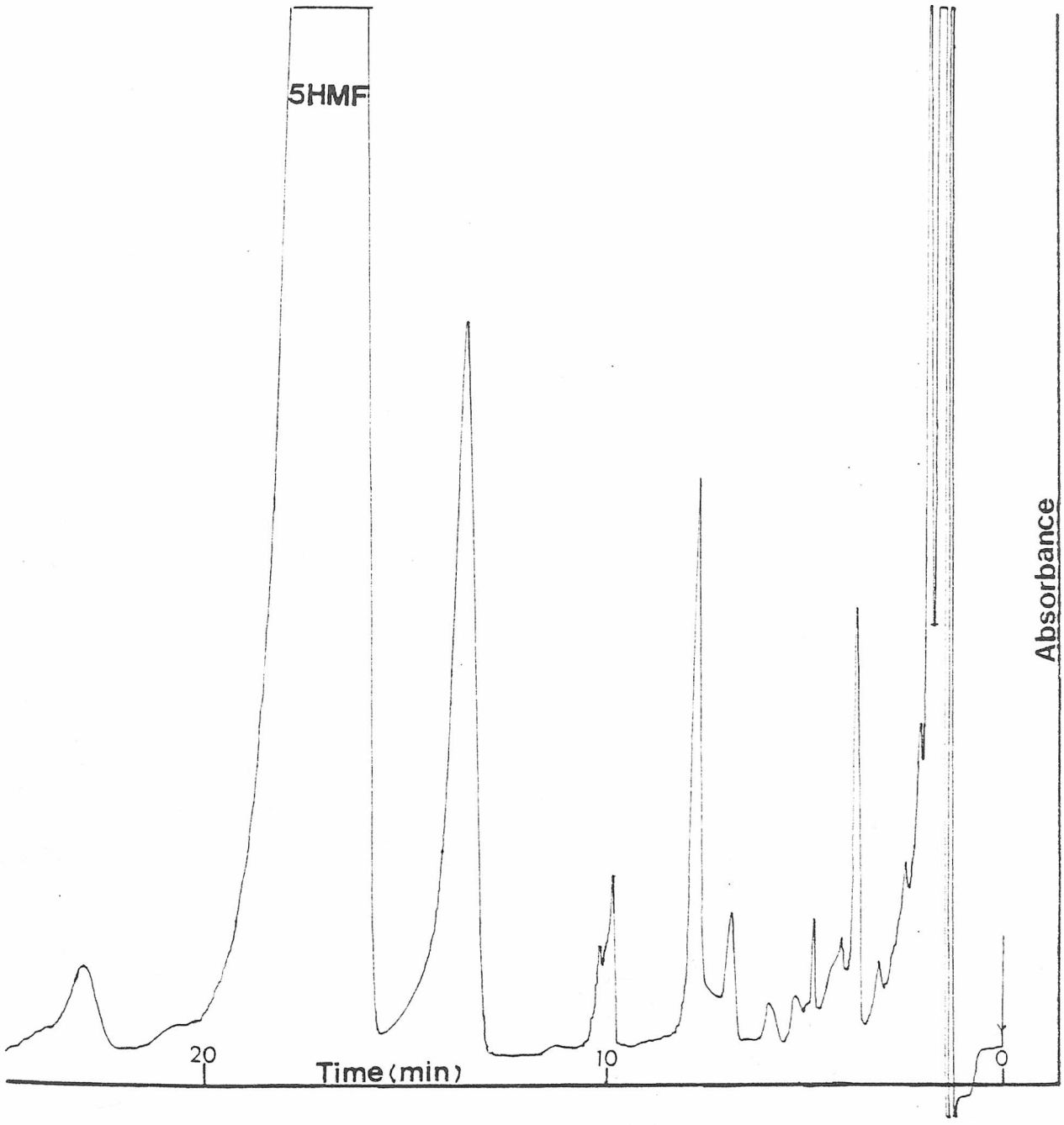


Figure 28 Chromatogram of degraded D-glucose solution initially adjusted to pH 2.5 before heating at 120°C. Chromatographic conditions as in Figure (18).

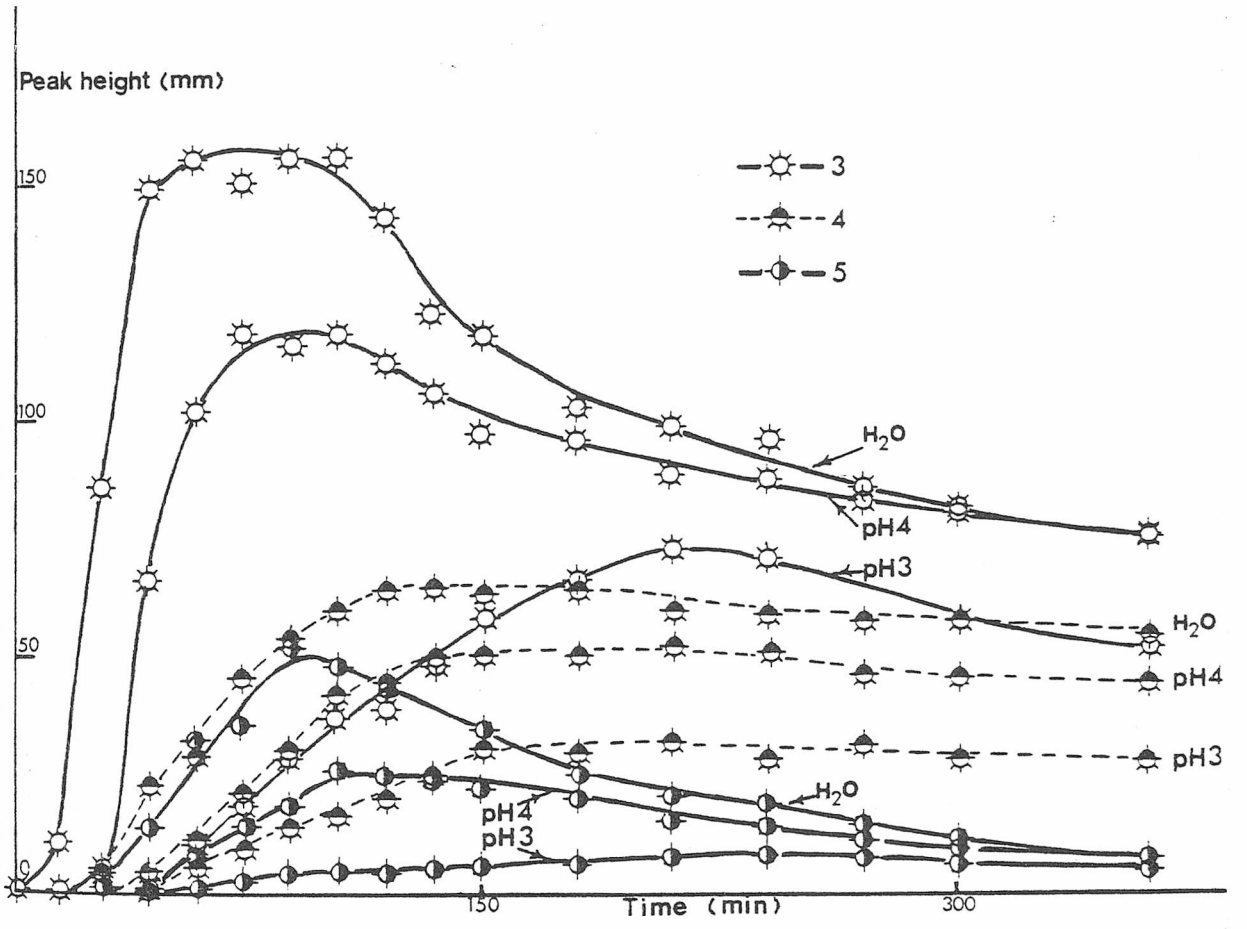


Figure 29a Variation of peak heights (corrected to full scale deflection of 0.04 absorbance units) of intermediates 3, 4 and 5 produced from D-glucose solutions initially adjusted to different pH values with time at 120°C. Chromatographic conditions as in Figure (18). Wavelength of detection of intermediate 4 was at 280 nm.

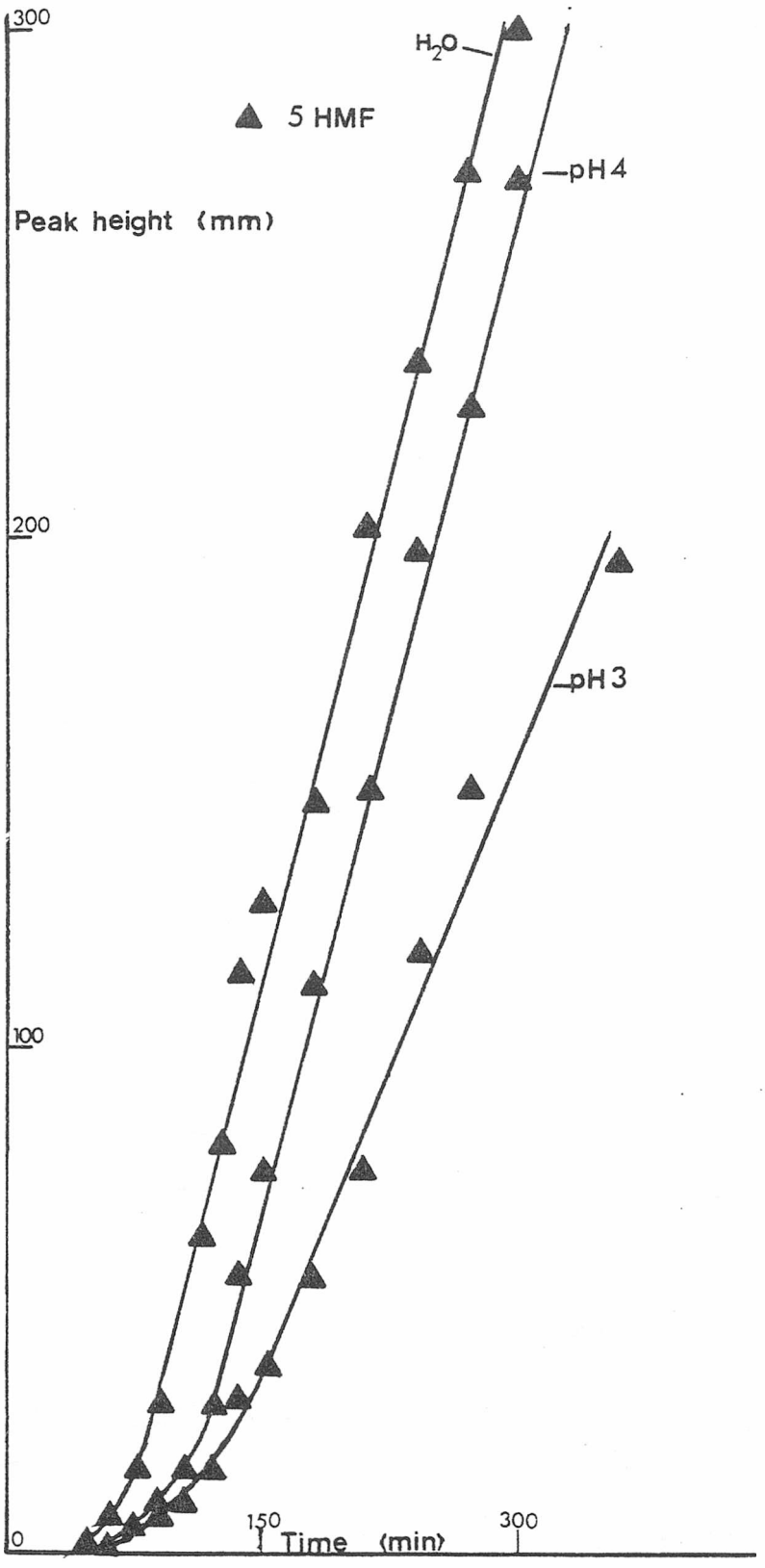


Figure 29b Variation of peak heights (corrected to full scale deflection of 0.04 absorbance units) of 5HMF produced from D-glucose solutions initially adjusted to different pH with time at 120°C. Chromatographic conditions as in Figure (18).

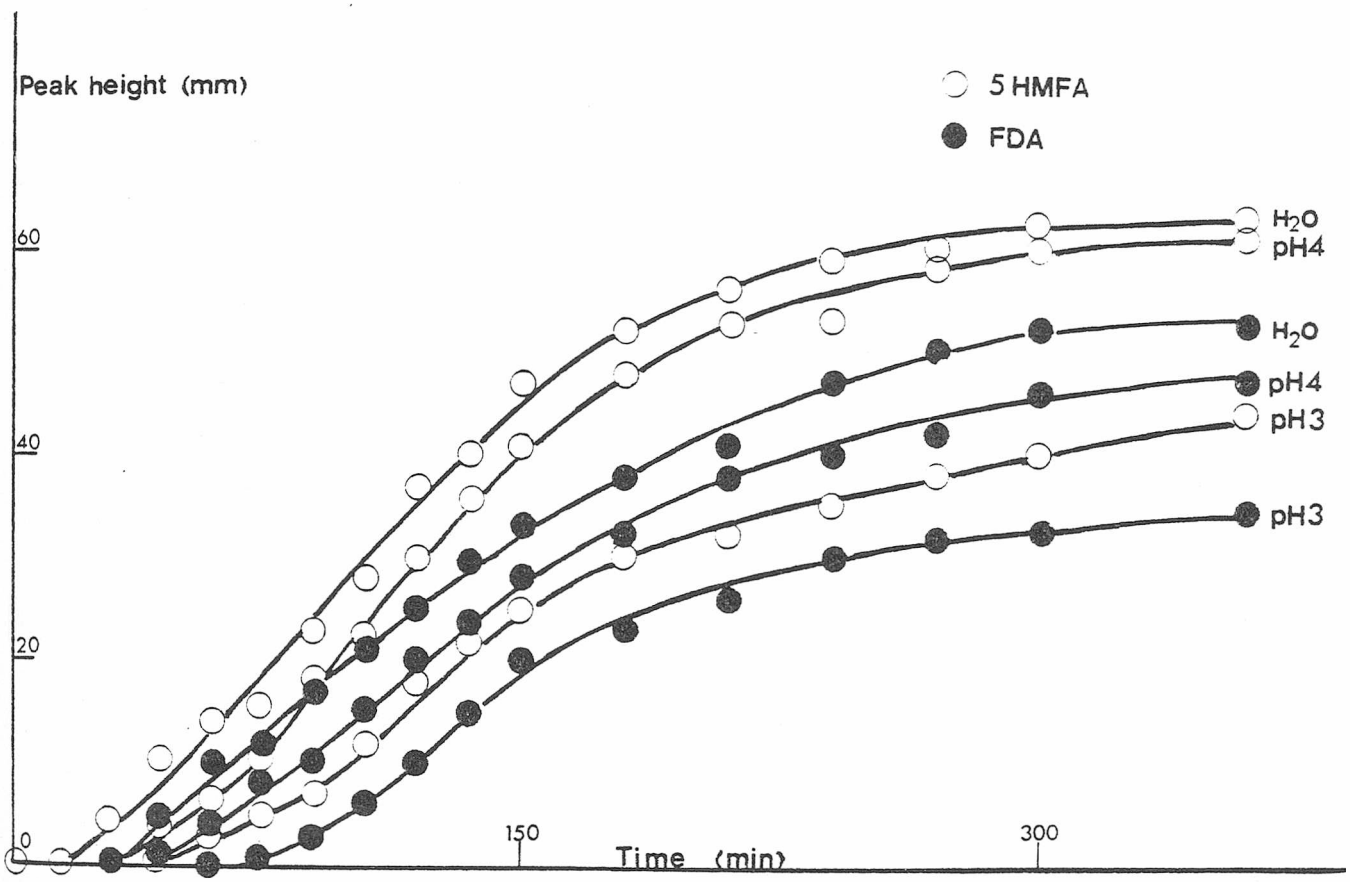


Figure 29c Variation of peak heights (corrected to full scale deflection of 0.04 absorbance units) of acids A and B produced from D-glucose solutions initially adjusted to different pH values with time at 120°C. Chromatographic conditions as in Figure (18).

CHAPTER FOUR

A STUDY OF THE EFFECT OF ADDED ORGANIC COUNTER ION IN THE ANALYSIS OF SEVERAL COMMONLY USED TRICYCLIC ANTIDEPRESSANT DRUGS IN SERUM USING REVERSED PHASE ION-PAIR HPLC

4.1 Introduction

The first so-called tricyclic antidepressant drug, imipramine, was introduced into clinical practice by Kuhn in 1958 [189]. After its clinical effectiveness had been demonstrated, many chemically related drugs were soon synthesised, and these compounds are now the most commonly used drugs in the treatment of depression [190]. The chemical structure of the major tricyclic and related drugs are outlined in Figure (30).

Although it has been generally agreed that these compounds are effective drugs, there are still many factors which can influence the success or failure of treatment. One of the most important factors is the diagnostic criterion used to select patients for drug therapy. It has been demonstrated that tricyclic antidepressants are most effective in treatment of endogenous depression, and are rather ineffective in reactive or neurotic depression [191].

The other major factor is the level of drug concentration actually achieved during treatment which may be affected by patient compliance and physiological factors [192-195]. On the other hand some workers have claimed that there is no significant correlation between the plasma antidepressant concentration and clinical response [196]. Others suggest that in common with other drugs, tricyclic antidepressants are effective only within a defined therapeutic concentration range [197-200]. Nonetheless, the importance, for therapy of a knowledge of drug concentration in blood rather than of size of the daily dose is becoming increasingly appreciated. Many antiepileptic and antiarrythmic drugs as well as theophylline and indomethecin are now routinely measured in serum in many clinical laboratories.

Tricyclic antidepressants which are both clinically and commercially important drugs have not been measured routinely in laboratories due to their low serum levels. Only recently has the advance of analytical technology enabled such low levels to be determined with accuracy.

Fluorimetric [201,202] and radio-immunoassay [203] techniques have been developed and have adequate sensitivity, but the lack of specificity because of metabolite interference makes these methods unattractive. The use of gas-liquid chromatography together with a sensitive detection system, such as electron capture [204] or nitrogen specific detectors [205,206] offers analytical methods with sufficient sensitivity and specificity. However, the procedures involved in making the derivatives for such analysis are time consuming and cumbersome. HPLC is obviously the alternative choice for the quantitative determination of tricyclic drugs. The procedures involved are usually quite straightforward and this technique should provide adequate specificity and sensitivity. The analysis of tricyclic drugs by HPLC is not without problems, however, these are usually associated with poor chromatographic efficiency due to the ionization of these compounds in the eluent [204].

Tricyclic antidepressants are basic compounds, with pKa values above 8 [208], which will ionise in the acidic or neutral solvents usually employed in HPLC. Ionic suppression techniques, which have been succesfully applied in the analysis of acidic products [73,124], will be less advantageous in the assay of tricyclic drugs due to the devastating effect on the silica support of the high pH values required for suppression [74,209].

Numerous articles have been published recently concerning the use of adsorption chromatography and reversed phase ion-pairing techniques [212,213] in the analysis of tricyclic drugs in which excellent results are claimed. Unfortunately, few of these publications have described the experimental conditions adequately enough for the results to be reproduced by other workers. The literature also contains several reports of organic amines being added to the mobile phase during the chromatography of basic drugs; in particular the tricyclic antidepressants [213-218]. The effect of such addition is generally to reduce tailing of eluted peaks. This has been

interpreted on the basis of masking active silanol groups of the C-18 silica, resulting in reduced heterogeneity of the stationary phase [219]. On the other hand the effect has also been explained on the basis of suppression of dissociation of ion-pairs in the organic phase [213,218].

In view of the ion-exchange-desolvation mechanism suggested in Chapter (2), any substance processing the same charge as the solute when added to the mobile phase will behave as a counter ion. The increase in counter ion concentration will act to reduce the retention of the solute. In Chapter (2) only inorganic species were considered as counter ions in which case interaction occurs mainly in the mobile phase. When organic amines are included in the mobile phase during the chromatography of tricyclic drugs, such added counter ion i.e. the amines, may not be confined to the mobile phase but may be absorbed to a certain extent onto the C-18 surface. Such a process will inevitably alter the properties of the stationary support. The use of ion-pairing chromatography with the concommitant addition of organic counter ion might have interesting effects on the retention behaviour of the ionic solutes.

Most of the data on such separations have been obtained in liquid-liquid systems in which higher alcohols are used to modify the C-8 or C-18 surfaces [213,215,218]. No information is available on the effect of such amine addition on the chromatography of basic drugs on modern system involving C-18 surfaces. Neither has there been any systematic study as to the relative effectiveness of different organic bases in improving the chromatography of basic solutes.

The ion-exchange-desolvation model as expressed in Equation (46) indicates that the retention of ionic solutes in ion-pair chromatography depends on the adsorbed pairing ion and mobile phase counter ion concentrations. The effects of different pairing ions on the retention of different ionic solutes have already been discussed in Chapters (2) and (3).

The effects of counter ion have so far been limited to the inorganic types and have been found to have only a slight effect in the reduction in retention [95,123,129]. The IEDM, indeed, assumes that the action of counter ion in the retention process is purely in the mobile phase. It should however, in principle, be possible to interpret the results obtained from chromatography including added organic counter ion on the basis of the processes believed to be responsible for retention during BRPIPC. Evaluation of the previous explanations for the improved chromatography of basic drugs which seems to result from such additions can also be made.

4.2 Scope of investigation

It was intended to examine the effect of several amines and quarternary ammonium salts having differing hydrophobicities on the chromatography of several tricyclic antidepressant drugs. It was also intended to attempt to explain the mode of action of such added amines in the ion-pair chromatography of basic solutes in the light of the ion-exchange-desolvation mechanism previously proposed in Chapter (2).

The other purpose of this investigation was to develop an assay method for the routine measurement of tricyclic drugs in blood serum particularly for the most widely used tricyclics, amitriptyline, nortriptyline, imipramine and desipramine. This group is particularly important because as with all other tricyclic drugs, amitriptyline and imipramine are known to metabolise in the body [220]. The corresponding metabolites nortriptyline and desipramine are also clinically active and thus must be accounted for in any monitoring scheme.

Controlled comparisons of the tricyclics have usually concluded that they are roughly equivalent drugs [221-222]. In practice, however, it is often noticed that individual patients may fare better on one drug

than on another, for reasons that are still uncertain. Finding the right drug for the patient is accomplished empirically at the moment [223]. So far, it is common practice to change a tricyclic drug, when it does not provide the desired response, to another tricyclic antidepressant that is chemically least similar to it. Recently, several so-called new generation tricyclic antidepressants have been introduced into practice, e.g. maprotiline and mianserine. Their structures bear little resemblance to the conventional tricyclics. Because of this they are the obvious alternative choices to replace the conventional tricyclic drugs when they are found to be clinically ineffective.

It was thus also an aim of this study to provide an assay method that could measure the serum concentration of maprotiline, since it has been used extensively for this purpose recently [224], when drugs such as amitriptyline and nortriptyline, or, imipramine and desipramine are also present in the serum. Such specific analytical measurements could provide valuable information for the physician in monitoring drug dosage of patients during the transient periods when maprotiline is being substituted for another drug.

4.3 Experimental

Chromatography was carried out using a variety of equipment including Altex (Model 110A) and Water Associates (M600A) constant flow pumps. Detection was by Cecil (CE 2012) and Pye-Unicam (LC3) detectors. Injection was by Rheodyne 7125 valve fitted with 20 or 100 µl loops. Columns were either 100 mm or 70 mm long and 4.6 mm internal diameter incorporating Swagelok end fittings and slurry packed with 5 µm ODS-Hypersil (Shandon Southern Products) as previously described in Section (2.3.3). Retention time data were measured directly from chromatograms as recorded on a Servocribe recorder.

Materials

The tricyclic antidepressants amitriptyline (Roche), nortriptyline (Lilly), imipramine (Geigy), desipramine (Geigy), doxepin (Pfizer), maprotiline (Geigy), mianserine (Bencard), nomifenxine (Hoechst), protriptyline (M.S.D.), trimipramine (M & B), clomipramine (Geigy) and dothiepin (Boots) were kindly donated by their manufacturers. The drugs were used as the hydrochlorides at a concentration of 50 $\mu g\ cm^{-3}$. Sodium laurylsulphate (SLS), dimethylamine (DMA) and triethylamine (TRIEA) were obtained from Fisons Ltd. Methylammonium chloride (META), trimethylamine (TRIMA), propylamine (PPLA) and buffer salt disodium hydrogen phosphate and diethylether were obtained from B.D.H. (Poole, Great Britain). Tetramethyl-(TMA), tetraethyl-(TEA) and tetrabutylammonium-(TBA) bromides were obtained from Aldrich Chemical Company Ltd. (Milwaukee, WI, U.S.A). Cetrimide-(CETA) was obtained from ICI Pharmaceuticals Ltd. (Macclesfield, Great Britain). Water used in chromatography was glass distilled and acetonitrile (HPLC grade) was obtained from Rathburn Chemicals Ltd. and Fisons Ltd. All other reagents were of AnalaR or similar grade.

Extraction of plasma samples

To 2 ml of serum in a 10 ml conical centrifuge tube, 500 μl of 1M sodium hydroxide and 5 ml of diethylether were added. The tube was shaken vigorously for 3 minutes. The organic layer was then transferred to another 10 ml conical centrifuge tube. The above procedure was repeated by further addition of 4 ml diethylether into the serum sample. 100 μl of 0.5 sulphuric acid was added to the approximately 9 ml of organic extract. The mixture was shaken vigorously for 5 minutes and centrifuged for 3 minutes at 3000 rpm. The organic layer was then discarded and the total aqueous extract injected into the chromatograph. Calibration curves for the five tricyclic antidepressants amitriptyline, nortriptyline, imipramine, desipramine and maprotiline using aqueous doxepine hydrochloride solution as internal standard were run in bovine serum by spiking to provide

concentrations in the range of 50–500 ng ml^{-1} for each compound. All measurements were made at ambient temperature.

4.4 Results and discussion

One of the most important requirements in a routine analytical method, beside the required sensitivity and specificity, is the reproducibility of results. In order to determine the optimal and easily reproducible chromatographic conditions for the analysis of the tricyclic antidepressants by HPLC, a number of experiments were carried out. In ion-pair chromatography, the retention time of the solute depends on the choice of the pairing ion and upon its concentration, and also the concentration of the organic modifier in the eluent. Their effects have been demonstrated in Chapter (2). In addition, the retention also depends on the pH and ionic strength of the mobile phase [73,124].

4.4.1 Influence of pH

Figure (31) shows the variation of k' of several tricyclic antidepressants as a function of pH in a mobile phase of 50% v/v acetonitrile water mixture. The retention of the drugs is observed to increase as the pH increases and such an effect has been demonstrated by many other workers [212,219]. That is at high pH, the degree of ionisation is decreased resulting in an increase of retention [124]. At low pH, the tricyclic drugs exist in fully ionised form and thus are eluted rapidly. It is observed from Figure (31) that at below pH3, the k' of the solutes is very insensitive to pH changes. Whilst at pH values above 3, especially between pH 6–7, slight variation will lead to significant changes in the k' of the solutes. Also, at high pH severe peak tailing effects were observed.

Other workers have claimed optimum separation of such compounds at pH 6.2–6.5 in presence of pairing ion [212]. It is possible that these

results, obtained with high organic modifier concentration and very low pairing ion concentration reflect the separation of the compounds partially ionised forms and are not significantly affected by pairing ion. In the present work a pH of 2 was selected. This had the advantages of least sensitivity of k' to pH for all solutes and also ensured that all amines added as counter ions would be present in the fully protonated state.

4.4.2 Influence of buffer salt concentration

Insufficient buffer capacity of the eluent in ion-pair chromatography will cause poor column efficiency and may result in peak splitting [239]. On the other hand, the addition of buffer salt into the mobile phase will alter its ionic strength which in turn has been demonstrated to have a significant influence on the solute retention [73,124,219]. The effect of phosphate buffer concentration on the retention of fully ionised tricyclic antidepressants is shown in Figure (32).

Surprisingly, Figure (32) shows that increase in the concentration of the disodium hydrogen phosphate causes a general decrease in the retention of the solutes. Such observations contradict previously reported results [73,124]. The decrease in retention of protonated basic substances with added electrolyte has been explained as due to the added salt competing for the free silanol groups on the C-18 surface [219]. This is because it is known that the free silanol groups on the silica surface will act as cation-exchange sites and cause retention of any basic solute [225]. However, such an explanation appears to be inadequate in the present study. This may be because ODS-Hypersil is a capped phase and has minimal free silanol groups. In addition, the pKa for such silanol groups as 9.5 [74], so that at the pH 2 used in the present measurements silanol ionisation and exchange would be expected to be negligible.

Figure (32) indicates that, although ionised salt concentration in general decreases retention, the effect is reduced above a concentration of 10 mM. Also it was observed that peak tailing decreased as the buffer salt concentration increased. For these reasons, eluents 10 mM in disodium hydrogen phosphate were used in the investigation. Higher concentrations were avoided in order to minimise the influence of the buffer as an inorganic counter ion so that any slight changes in the retention behaviour of the solute on addition of organic amine could be observed. Moreover, 10 mM phosphate buffer can provide adequate buffer capacity and very reproducible retention data.

4.4.3 Influence of acetronitrile

Figure (33) shows the effect of the acetonitrile content of the eluent on k' of the solutes. An increase in the acetonitrile concentration decreases the retention with some changes in elution order and lowers the selectivity factors. At below 50% v/v acetonitrile, slight variation of the organic modifier content causes marked changes in k' and also peak tailing becomes apparent. At higher acetonitrile content selectivity factors become minimal. In addition, at very high organic modifier concentration, loading of a pairing on the C-18 surface will be reduced (Section 2.3.4) and this will limit the use of pairing ion concentration to govern the retention of the solutes. 50% v/v was therefore used as the content of organic modifier in this investigation.

4.4.4 Influence of pairing ion

Due to the use of 50% v/v acetonitrile water buffer as the eluent, sodium laurylsulphate was employed as the pairing ion. This is because it is a very hydrophobic pairing ion and has been demonstrated previously (Section 2.3.4) to have appreciable adsorption on the C-18 surface even at such high organic modifier concentration. Other pairing ions of lower

hydrophobicity would produce smaller k' values, but would result in no significant improvement in resolution among the solutes.

The separation obtained among twelve tricyclic antidepressants is summarised in Figure (34). It shows that the k' values of all solutes go through the expected maxima with pairing ion concentration and that for most solutes maximum retention and also the optimum separation is achieved at a SLS mobile phase concentration of 80 mM. While considerable separation is obtained among several of the test compounds at the maximum in k', the clinically relevant separations namely between amitriptyline and nortriptyline and between imipramine and desipramine are not adequate. The separations achieved are further demonstrated in Figure (35A-C) which shows representative chromatograms of selected groups of drugs among the compounds in the class. It also indicates that good chromatographic peak symmetry is achieved with little evidence of the peak tailing problems claimed to be associated with such compounds even when ion-pairing techniques are used [213,218].

Since increasing pH, in absence of pairing ion appears to increase selectivity, Figure (31), the solutes were chromatographed at pH 6.5 using an eluent 80 mM in pairing ion, 10 mM in buffer and with an organic modifier concentration of 50% v/v. No improvement in resolution between the clinically important pairs amitriptyline and nortriptyline nor between imipramine and desipramine was observed. On the other hand, compounds which can be separated using the system as demonstrated in Figure (35) were seen to have poor resolution when operated at pH 6.5 as shown in Figure (36). The poor resolution at high pH appeared due to the peak tailing problems. The problem of peak tailing at high pH may be due to the retention behaviour of the neutral tricyclic bases differing from that of their protonated forms. This is consistent with the slightly increased times shown at high pH by other workers [238]. No alteration or improvement in selectivity is

apparent in Figure (36).

4.4.5 Influence of organic amines

Various concentrations of different organic basic salts were used to evaluate their effects in the ion-pair chromatography of basic solutes. Organic counter ions ranging in hydrophobicity from methylammonium chloride to cetrimide were added into the mobile phase of 80 mM SLS in 50% v/v acetonitrile buffer. Figure (37) shows the effect of such counter ion addition on a given test solute, amitriptyline. Their effect is to decrease the retention of the solute. This was found to be general for all other tricyclic antidepressants but the extents of the decrease differed. Figure (37) also points out that the effect is greater the higher the hydrophobicity of the added counter ion. However, the inverse relationship between k' and counter ion concentration characteristic of inorganic counter ions [104,123] is not evident in the present work with hydrophobic counter ions. When the results shown in Figure (37) are plotted according to the generally accepted role of counter ion in the ion-exchange models of retention i.e. k' as a function of the reciprocal of the counter ion concentration, the resulting lines are shown in Figure (38a). These are seen to be in agreement with the reciprocal relationship only at very low concentration. In the case of CETA the relationship is not obeyed even at concentrations of 0.1 mM. For the other organic counter ions there is marked non-linearity of these curves at concentrations above 10 mM. The corresponding plot for sodium as an inorganic counter ion is shown in Figure (38b). It can be seen that the behaviour pattern shown for the organic species is similar to that for sodium acting as counter ion. In the case of sodium, however, there is a marked linear region at concentrations between 20 and 150 mM. Above this concentration a similar non-linearity is observed and such non-linear plots have also been demonstrated by Tomlinison et al. [101]. It would appear from these results that

similar effects are operating for both organic and inorganic counter ions.

The ion-exchange mechanism responsible for the prediction of the reciprocal relationship considers only interaction in the mobile phase. It may be that the counter ion directly affects the adsorption properties of the pairing ion. If such is the case it would appear from these results that the addition of pairing ion at high counter ion concentrations exerts a disproportionate effect on k' which might be explained by competition for C-18 surface between pairing ion and counter ion. This is contrary to the effect noted by Van de Venne and co-workers [124] where an increase in counter ion concentration has been shown to produce a slight increase in adsorbed pairing ion concentration. The present results do indicate that the action of counter ion is more complex than that envisaged in classical ion exchange chromatography and that some interaction with the stationary phase must occur. This is confirmed by the relative magnitudes of the decreases observed with different counter ions. At comparable concentrations the more hydrophobic the counter ion the greater does the decrease in k' seem to be.

It is also noted that while peak sharpening occurs on the addition of organic counter ions, it is as a consequence of the decreased retention time. No real increase in plate number is obtained as a result of organic counter ion addition. One striking effect of the added organic counter ion on the chromatography of the tricyclic drugs, however, is the resolution between the pair amitriptyline and nortriptyline, as shown in Figure (39). The effect of addition of amine to the eluent is to increase the resolution of this pair of compounds. The improvement is most marked in the case of cetrimide and least for methylammonium chloride. The effect of inorganic counter ion was observed to be minimal. These effects were also observed for the imipramine, desipramine pair of drugs.

Although cetrimide appears to be the most effective counter ion to

improve the degree of resolution obtainable, and also requires the minimum concentration to achieve this, the retention times become very short, Figure (40A-C), and thus unacceptable for application to assay situations. Also, slight variations in the added cetrimide concentration will cause substantial changes in the k' values of the solutes.

Tetrabutylammonium (TBA) ion provides a better compromise between retention time and resolution for the above pairs of compounds. Representative chromatograms are shown in Figure (41A-C), demonstrating the separations that can be achieved among selected members of the tricyclic antidepressant group of compounds by the addition of 5 mM TBA. From the results as shown in Figures (35) and (41), it is noticed that although that peaks in presence of 5 mM TBA are considerably sharper the retention times have also been decreased and, in fact, measured plate numbers are similar. It is also observed that the resolution among the compounds has changed. Compounds previously separable without addition of added counter ion are now unresolved and vice versa. The retention data of all twelve tricyclic drugs as a function of mobile phase pairing ion concentration in presence of 5 mM TBA are shown in Figure (42). The maximum in k' with pairing ion concentration is still apparent but the relative magnitudes of the capacity factors at the maximum are drastically altered.

The improvement in chromatographic separation of basic drugs as a result of added amine has been suggested as being a consequence of decreased peak tailing due to inactivation of unreacted silanol groups [219] or alternatively as a result of suppression of secondary equilibria, namely the dissociation of ion-pairs adsorbed on the C-18 surface [218]. Both of these explanations imply that the improvement in separations of all basic solutes is due to an increase in column efficiency.

Any increase in efficiency in such systems involving added amine

would be as a result of an increased rate of mass transfer. The mass transfer coefficient, measured as the slope of the HETP against mobile phase linear velocity curve, was compared in presence and absence of 5 mM TBA for four solutes in a mobile phase of acetonitrile buffer mixture containing 80 mM SLS. Figure (43) shows such a plot for amitriptyline. The variation of plate height for nortriptyline, imipramine and desipramine as a function of mobile phase linear velocity is shown in Table (6). No significant alteration in gradient was observed on addition of amine for any of the four solutes. This was taken as indicating constancy in the mass transfer rate and as further verifying the lack of real increase in column efficiency.

While the efficiency of the column is unaffected by addition of organic counter ion, resolution among the different solutes is significantly altered. The factors contributing to resolution between a pair of compounds are included in Equation (55),[226]

$$R = \frac{1}{4} \cdot \left(\frac{\alpha - 1}{\alpha}\right) \cdot \left(\frac{k'}{1 + k'}\right) \cdot N^{\frac{1}{2}} \qquad -(55)$$

where α, k' and N are as defined in Section (2.1). In this present investigation, N is constant and the effect of added amine is to reduce k' so that any increase in R would appear to be as a consequence of a change in the selectivity term $(\alpha - 1)/\alpha$. The variation of $(\alpha - 1)/\alpha$ between amitriptyline and nortriptyline with concentration of various counter ions is shown in Figure (44) and the overall shape of the plots appear to parallel that of the resolution as shown in Figure (39).

The ion-exchange-desolvation model formulated for hydrophobic pairing ions on octadecylsilica using aqueous mobile phases can be used to explain the present findings. This model envisages retention of a basic solute in a completely ionised form by a hydrophobic anionic pairing ion adsorbed to an equilibrium extent on the C-18 surface. The ionised solute competes

with the counter ion for the adsorbed pairing ion and subsequently desolvates onto the C-18 surface when its charge has been neutralised. In the present study the hydrophobic nature of the counter ions will modify the behaviour from that observed with inorganic counter ions such as buffer salts, the interaction of which with the stationary phase pairing ion is largely electrostatic. When counter ions differing in hydrophobic character are used, the counter ion, as well as being bound to the pairing ion by columbic forces, is also desolvated on the adjacent C-18 surface. This equilibrium situation is identical in nature with the transient equilibria, producing retention of any hydrophobic solute. For retention of a solute to be achieved when organic counter ions are employed, not only must ion exchange take place between solute and counter ion, but the counter ion must be resolvated into the mobile phase. Thus, the more hydrophobic the counter ion, the more difficult will the resolvation process be and the greater will be the reduction in retention for a given concentration of added counter ion. Figure (37) is interpreted on this basis as representing the effect of gradual replacement of inorganic counter ion by organic amine. The equilibrium reaction producing retention in the situation involving organic counter ion may be represented as before, by the ion exchange reaction,

$$(P^-C^+)org + (A^+)aq \overset{K_{IE}}{\rightleftharpoons} (P^-A^+)org + (C^+)aq \qquad -(56)$$

where $(P^-C^+)org$ represent the adsorbed pairing ion together with its associated counter ion and (A^+) represents the fully ionised solute. The equilibrium constant, K_{IE}, will depend upon which species predominates as the counter ion, (C^+). That is, K_{IE} will vary from a maximum when the organic counter ion concentration is negligible to a minimum when all of the inorganic counter ion has been replaced by the added amine. Thus two limiting forms of the above equilibrium may be written,

$$(P^-Na^+)org + (A^+)aq \xrightleftharpoons{K_{IE}} (P^-A^+)org + (Na^+)aq \ (K_{IE} \text{ large}) \qquad -(57)$$

$$(P^-TBA^+)org + (A^+)aq \xrightleftharpoons{K_{IE}} (P^-A^+)org + (TBA^+)aq \ (K_{IE} \text{ small}) \qquad -(58)$$

At intermediate conditions, where not all of the sodium has been replaced due either to the limited hydrophobicity of the added amine, or to its low concentration, the value of K_{IE} will be intermediate between these extremes.

The equation derived previously relating capacity factor to adsorbed pairing ion and aqueous counter ion concentration, will still apply in the presence of both organic and inorganic counter ions, as long as the eluent counter ion concentration is held constant,

$$k' = \frac{1}{V_m} \cdot \left(As.K_{des} - K_{des}.[P^-C^+]org.Ap + K'_{des}.K_{IE}.\frac{[P^-C^+]org}{[C^+]aq} - K'_{des}.K_{IE}.Ap.\frac{[P^-C^+]^2org}{[C^+]aq} \right) \qquad -(46)$$

This form of equation is seen to apply both in the situation of pairing ion and buffer only for all the tricyclic antidepressant drugs investigated as shown by Figure (34) and also for the case of a fixed concentration of TBA as organic counter ion in presence of SLS pairing ion as shown in Figure (42). In this equation in the case of organic counter ion the $K'_{des}.K_{IE}$ term will not only reflect the desolvation of a given solute subsequent to ion-exchange but it will provide an estimate of the desolvation tendency of a solute displacing a bound organic counter ion. This is seen as providing an additional parameter of selectivity in such systems and may account for the alteration in elution order in Figure (42) compared with Figure (34). It is emphasised that while in this case the selectivity alteration has been to improve the resolution for the pairs desipramine and imipramine and amitriptyline and nortriptyline, its effect is also to reduce the resolution between other pairs as shown in Figure (41C) for

desipramine and amitriptyline. That is, the effect of the added amines is to reshuffle the positions of the peaks without altering the peak capacity.

The applicability of the above equation on a quantitative basis for the above pairs of compounds can be assessed by determining the $K'_{des}.K_{IE}$ constants for each compound in presence and absence of organic counter ion. The $K'_{des}.K_{IE}$ constants were evaluated as described in Chapter (2). $[P^-C^+]org$ values were obtained by interpolation of values from previous isotherms. Section (2.3.4.1), measured at 30% and 60% acetonitrile concentration and it is assumed on this model that area available for desolvation of solutes will not be affected by the presence of the more bulky organic counter ion. This is because for every solute desolvated on the C-18 surface, the associated inorganic or organic counter ion must be resolvated. Thus, the area available for desolvation of the solutes can be regarded as the total surface of the C-18 silica minus that area occupied by the pairing ion. If the equation is applicable to such systems involving added counter ion, the numberical values of $K'_{des}.K_{IE}$ should be seen to decrease in the presence of organic counter ion. In addition, the separation factors, (α), which can be measured by direct observation of the relative retention times between pairs of compounds should be identical with the ratio of calculated $K'_{des}.K_{IE}$ values for that pair of compounds obtained by the above curve fitting procedure. The results of such a comparison are shown in Table (7). It is seen that the expected decrease in $K'_{des}.K_{IE}$ is observed and that good agreement is obtained for the situation where resolution has been improved and for that in which resolution has been decreased. The alteration of selectivity of the stationary phase due to the effect of bound inorganic counter ion in ion-pair chromatography has also recently been reported by Deelder et al.[153] and has been explained on the basis of the adsorption of inorganic counter

ion within the electrical double layer.

The potential of counter ion programming in the gradient separation of ionic species using conventional ion-exchange columns has been demonstrated by numerous workers [111,227]. However, such a technique has not been applied to the improvement of separation of inogenic solutes in reversed phase ion-pair chromatography. In view of the effect of the added organic counter ions in ion-pair reversed phase HPLC, organic counter ion programming in the elution of ionic solutes may prove useful. The added organic counter ion will not only reduce the retention time of the solutes, as does an inorganic counter ion, but it will also alter the selectivity of the stationary phase as its concentration varies. With a suitable choice of organic counter ion, such a programming technique might provide a powerful tool in the separation of a large number of ionogenic solutes of widely differing, or of similar structures.

One other finding in this investigation is that any pairing ion commonly used in ion-pair chromatography can also be employed as a counter ion to alter the resolution of solutes, when a pairing ion of opposite charge is used in the chromatography.

4.4.6 Quantitative aspects of the method

In order to separate the clinically important pairs amitriptyline and nortriptyline and imipramine and desipramine the mobile phase system of 80 mM SLS, 5 mM TBA and 10 mM Na_2HPO_4 in 50% v/v acetonitrile water mixture adjusted to pH 2 was used. This provides the required selectivity and sensitivity for measurement of these drugs in serum at therapeutic levels.

Extraction procedure:

The liquid-liquid extraction methods described above Section (4.3), were found to be suitable for routine applications in terms of recovery,

and reproducibility. The extraction of the basic drugs from the alkali adjusted serum into the organic solvent followed by back extraction into dilute acid also acts as a sample cleaning procedure. The acidic and most of the neutral interfering substances can be eliminated from the sample by use of such extraction methods and a clean extract results. Chromatograms of a serum blank extract recorded at 254 and 230 nm are shown in Figure (45A-B).

Several organic solvents were evaluated for extraction namely ether, hexane and hexane/isoamyl alcohol mixtures. Ether offered the best compromise with respect to extraction efficiency and selectivity. When hexane was used as the solvent, a very clean extract was obtained but the recovery of tricyclic drugs was rather poor, usually between 50-70%. Whilst hexane/isoamyl alcohol extract at 99:1 ratio gave very good recoveries, above 70%, but with increasing background interference in the blank and with ready emulsion formation during the extraction.

Serum determination:

Internal standard was added into the serum sample prior to extraction in the serum determination of the tricyclic drugs. The use of an internal standard served to standardise the extraction procedure and also to eliminate any error measured due to incomplete injection of the total sample available.

In this study doxepin was employed as the internal standard. Its chemical structure is very similar to that of amitriptyline and imipramine, and also doxepin is rarely prescribed in this region.

In view of the ultraviolet absorption maxima of the six tricyclic drugs being investigated; 210 nm for doxepin, 240 nm for amitriptyline and nortriptyline, 250 nm for imipramine and desipramine and 220 nm for maprotiline, detection at a single fixed wavelength e.g. 254 nm seemed

inappropriate. Detection at 254 nm although less than optimal for amitriptyline and nortriptyline can still provide sufficient sensitivity for these drugs. However, in case of maprotiline, detection at 254 is quite inadequate to detect concentrations at the clinical level. Thus calibration curves of peak height ratio of the drug under study to the internal standard against concentration of drug were recorded using wavelengths of 254 nm and 230 nm. These results are discussed below.

When the detection wavelength is 254 nm, internal standard doxepin, is added into the sample at a concentration of 150 ng ml^{-1} of serum. Whilst when 230 nm is employed, the concentration of internal standard was 50 ng ml^{-1} of serum. HPLC traces of serum extracts of drugs, measured at wavelength of 230 nm are shown in Figure (46A-B). The tricyclic drugs are well resolved and well removed from endogenous plasma constituents. The corresponding results at 254 nm are identical except that maprotiline can not be detected at the level used.

Linearity:

Figure (47) shows calibration curves, obtained by plotting the ratio of the peak heights of each of the four tricyclic antidepressants to that of the internal standard, doxepin, against their corresponding concentrations at 254 nm. The calibration curve of maprotiline at 254 nm is not shown due to the poor sensitivity of this compound at this wavelength of detection.

The same plot of each of the five compounds i.e. amitriptyline, nortriptyline, imipramine, desipramine and maprotiline with detection at 230 nm are shown in Figure (48A-B). The relation is linear over this entire range. Table (8) and (9) lists the constants of the respective linear regression lines.

Reproducibility:

The precision of the method was assessed by repeated analysis of serum samples containing two different concentrations of all tricyclics studied corresponding approximately to the lower and upper limit of the therapeutic ranges reported for most of the tricyclic drugs. As shown in Table (10), at a wavelength of detection of 254 nm within day coefficients of variation varied between 1.9% and 5.4%, and day-to-day precision between 4.5% and 7.2%. Generally, the assay is more precise at high concentrations because recovery is more complete. Corresponding results when the detection wavelength was 230 nm are shown in Table (11). The precision varied between 3.2% to 7.3% within day and day-to-day between 6.2% to 10.3%. Although the precision at 230 nm us still within the generally accepted limits for drug assays, the coefficient of variation is significantly higher than that obtained at 254 nm. 254 nm is thus more advisable as the wavelength of detection in routine measurement, even though higher sensitivity can be obtained when measured at 230 nm, unless the determination of maprotiline serum concentration is required.

To further demonstrate the precision and accuracy of this method, it was used to analyse specimens of pooled serum containing either amitriptyline and nortriptyline, or imipramine and desipramine distributed by a nationwide laboratory test scheme [228]. In this scheme, every month specimens of known concentration of drugs are supplied to clinical laboratories participating in the analytical technique for the analysis of the samples. The results obtained from various laboratories and the spike values of the samples are then compared.

Table (12) lists the values of the spike serum concentrations of the specimens, the mean concentrations obtained by different laboratories using different assay methods and the concentration obtained by this investigation. Since very widely different coefficients of variation are obtained in such

laboratory test schemes [228-230], the results shown in Table (12) indicate that the assay method in this study is adequately accurate.

Recovery:

The absolute analytical recoveries from serum of five drugs and internal standard were measured at four different concentrations ranging from 50 to 400 ng ml^{-1} in the following way. The serum drugs were added to the blank serum at the concentration shown in Table (13). The serum concentration was then analysed by use of the same method but without internal standard. Absolute recovery was calculated by comparing these peak heights with those obtained by direct injection of the pure drug standards at the appropriate concentration. The results are shown in table as mean percentage recoveries.

The recoveries of all drugs are demonstrated to increase with increasing in concentration and such findings are consistent with those reported by other workers [212,231].

Detection limit:

At the highest detector sensitivity (0.005A full scale), under the condition of this assay the limit of detection for amitriptyline and nortriptyline was estimated as 2 ng ml^{-1} at 230 nm and 5 ng ml^{-1} at 254 nm taking a signal/noise ratio of 3 [212] as adequate. For imipramine and desipramine, this limit was estimated to be 5 ng ml^{-1} at either wavelengths of detection and that of maprotiline was determined as 10 ng ml^{-1} at 230 nm.

4.5 Conclusions

The present investigation using the closely related group of tricyclic antidepressant drugs as a model system indicates that the action of an organic amine when added to the mobile phase containing hydrophobic anionic pairing ion, SLS, is that of a counter ion involved in an ion-exchange-desolvation process. Such species, because of their hydrophobic nature

will desolvate on the C-18 surface and thus act to reduce the capacity factors of all basic solutes. The more hydrophobic the counter ion, the greater is its effect in reducing the retention of the solutes. Such counter ions may also provide an additional degree of selectivity over that observed in such system in absence of added amine. This effect is utilised to increase resolution among amitriptyline and nortriptyline, imipramine and desipramine. However such an effect may also act to reduce resolution among others. It has been found that the systems discussed can provide the required selectivity and sensitivity for the measurement of several commonly used tricyclic antidepressants in serum at therapeutic levels. An extraction method of tricyclic drugs from serum has been described, which requires only 20 minutes. This assay method has been studied for routine application in the clinical laboratory and has been proved to be satisfactory.

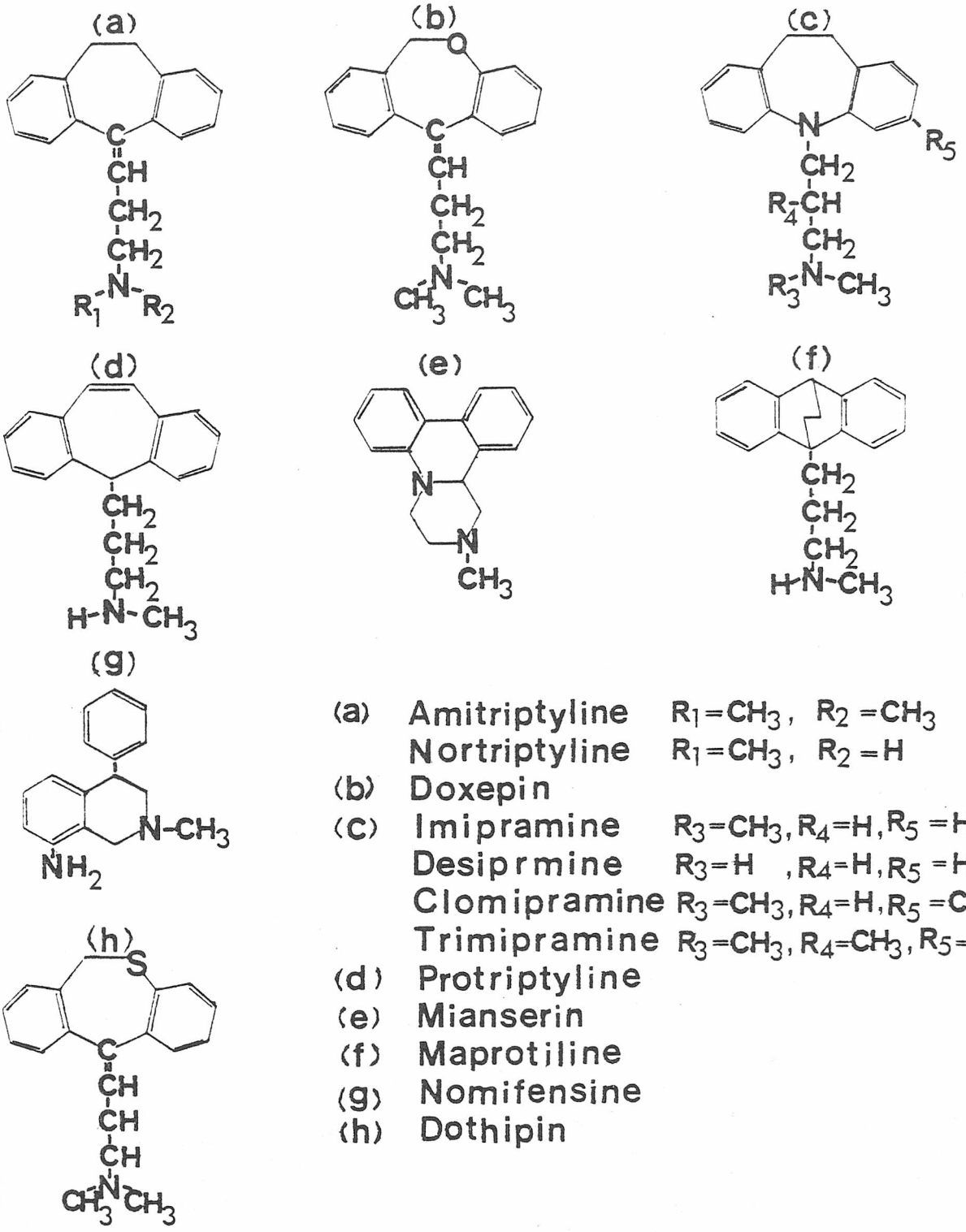


Figure 30 Chemical structures of the major tricyclic anti-depressant drugs.

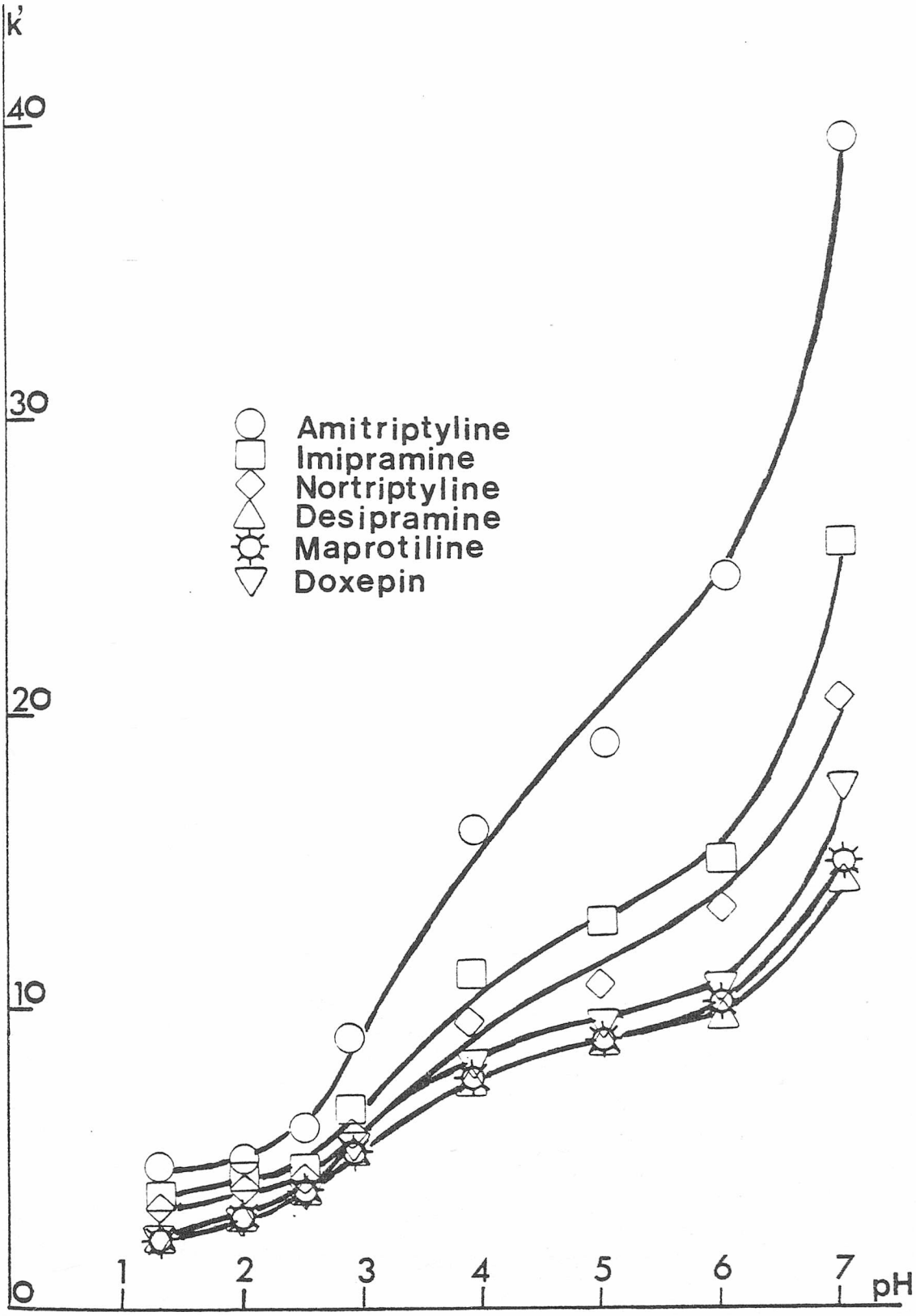


Figure 31 Plots showing the effect of pH on capacity factor (k') of some tricyclic antidepressants. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil column at 2.0 cm^3 min^{-1} flow rate; mobile phase of 50% v/v acetonitrile-buffer of 10 mM disodium hydrogen phosphate.

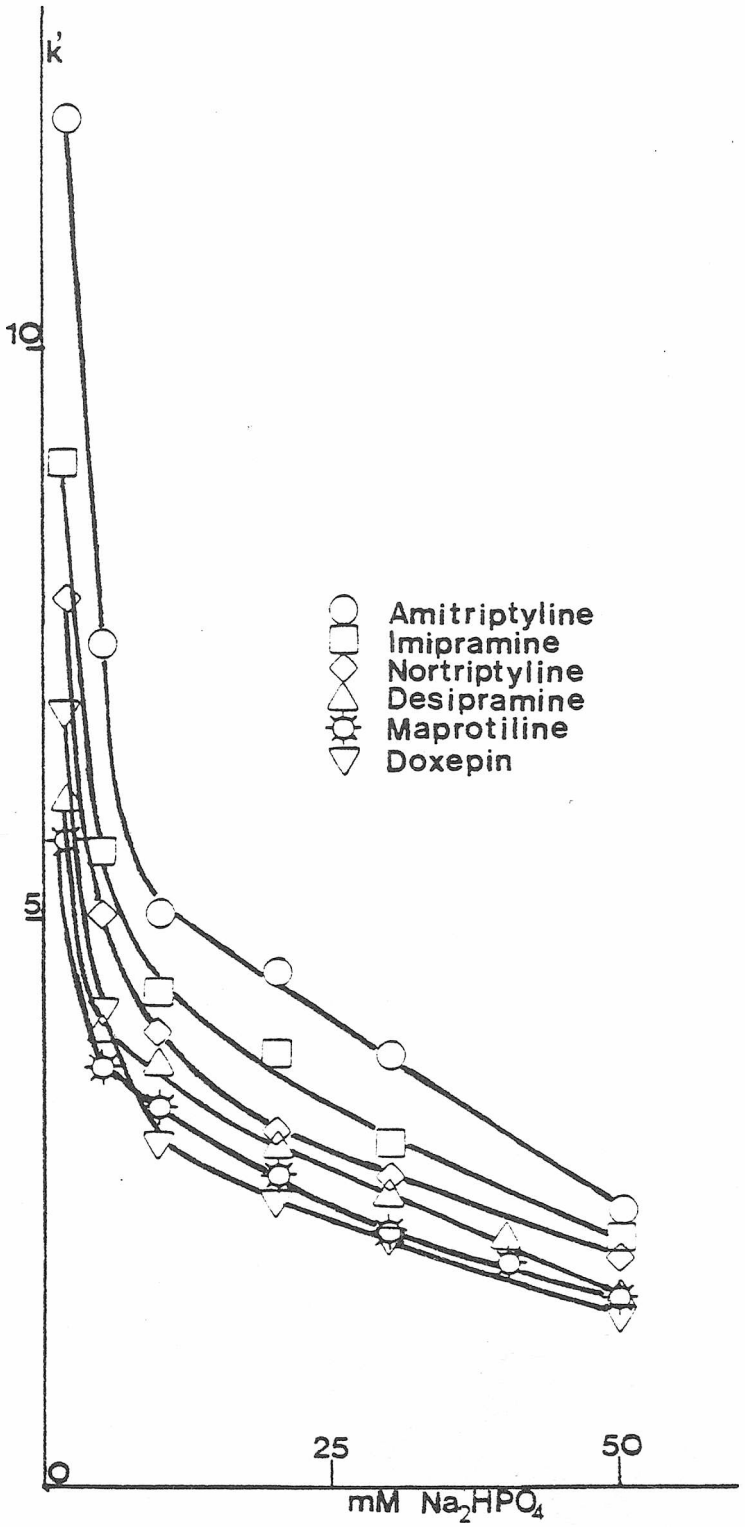


Figure 32 Effect of the buffer salt disodium hydrogen phosphate concentration on capacity factor (k') of tricyclic antidepressants. Chromatographic conditions: Column 100 x 4.6 mm, solvent 50% v/v acetonitrile–buffer at pH 2, flow rate 2.0 cm^3 min^{-1}.

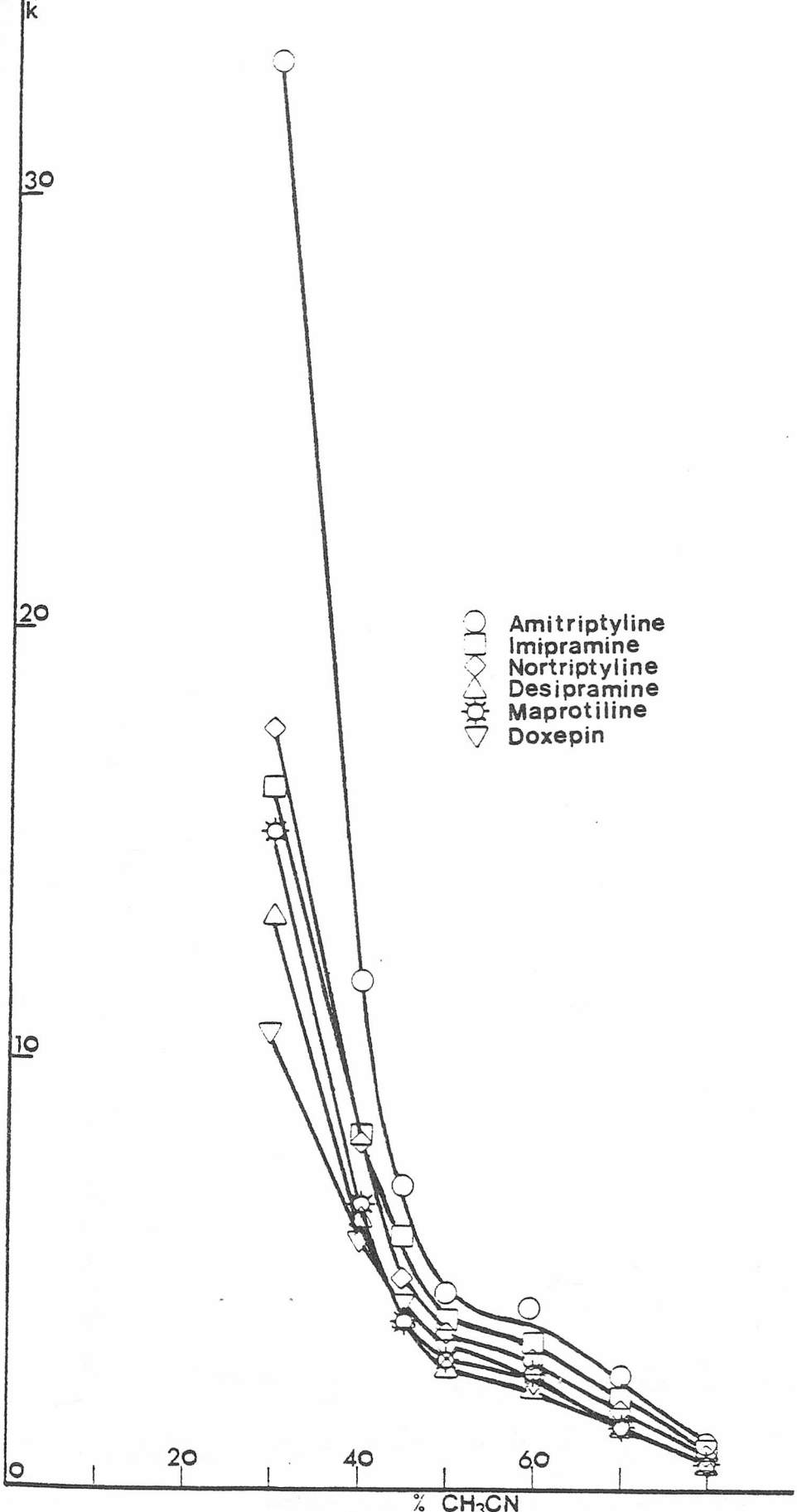


Figure 33 Influence of the acetonitrile content of the eluent on capacity factor (k') of tricyclic antidepressants. Chromatographic conditions: 100 x 4.6 ODS-Hypersil column; solvent acetonitrile-buffer of 10 mM disodium hydrogen phosphate at pH 2; flow rate 2.0 cm^3 min^{-1}.

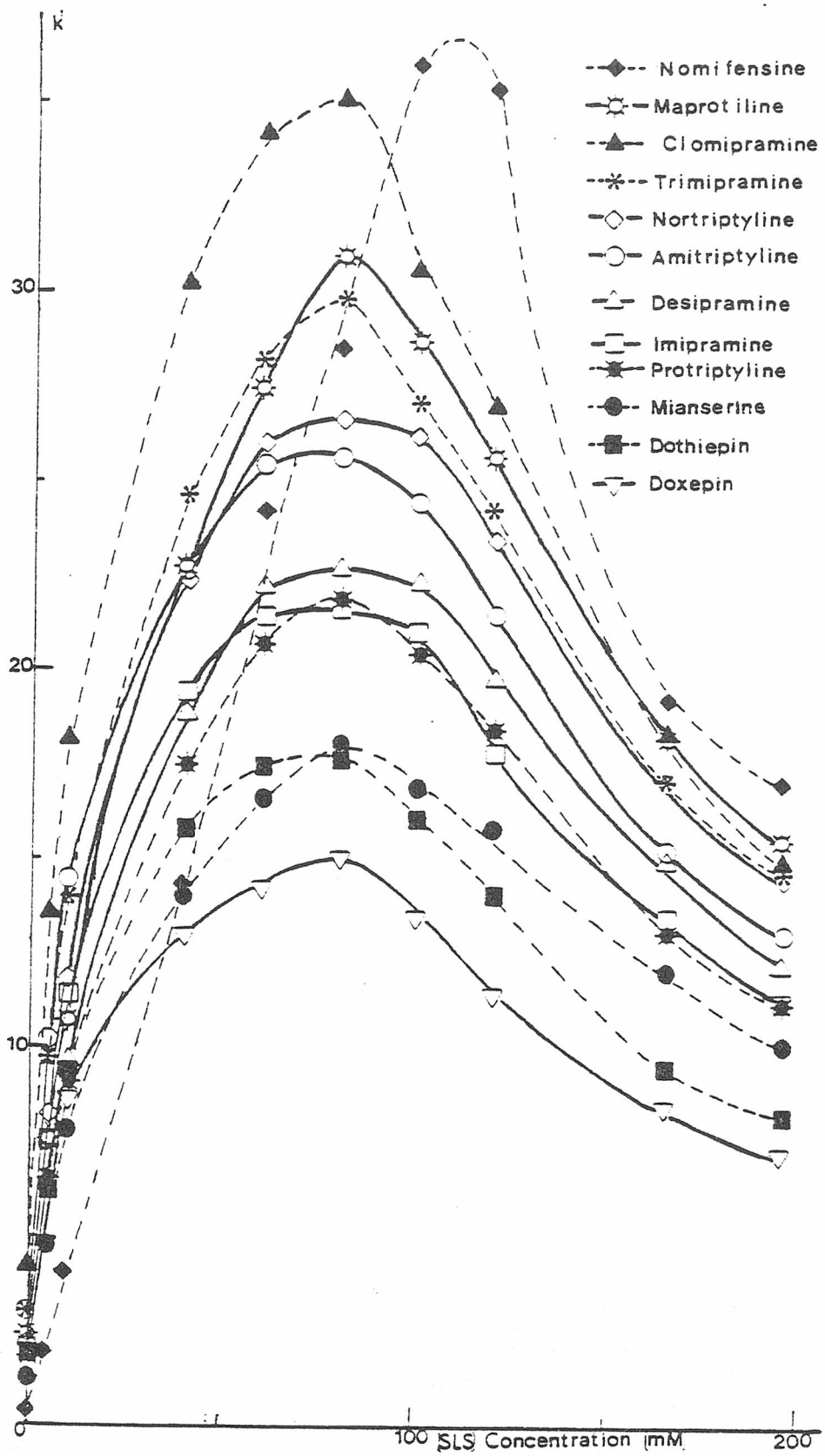


Figure 34 Plots showing the variation of capacity factor (k') with mobile phase pairing ion (SLS) concentration for twelve tricyclic antidepressants. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil column; mobile phase 50% v/v acetonitrile-buffer of 10 mM disodium hydrogen phosphate at pH 2. Flow rate 2 cm^3 min^{-1}.

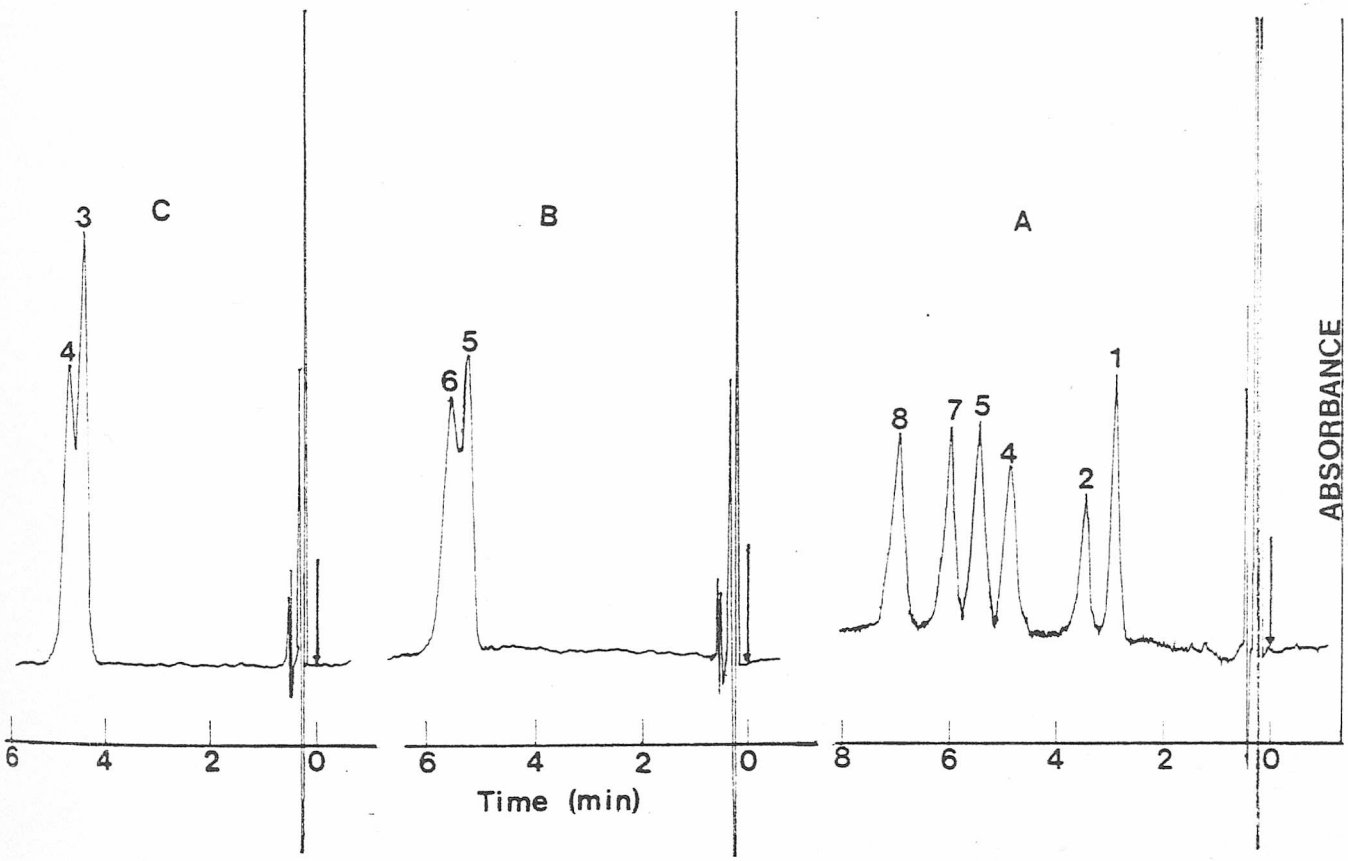


Figure 35 Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs. Chromatographic conditions: Column 70 x 4.6 mm, mobile phase as in Figure (34) with (SLS) concentration 80 nM. Compound identification 1 - doxepin, 2 - mianserine, 3 - imipramine, 4 - desipramine, 5 - amitriptyline, 6 - nortriptyline, 7 - trimipramine, 8 - clomipramine.

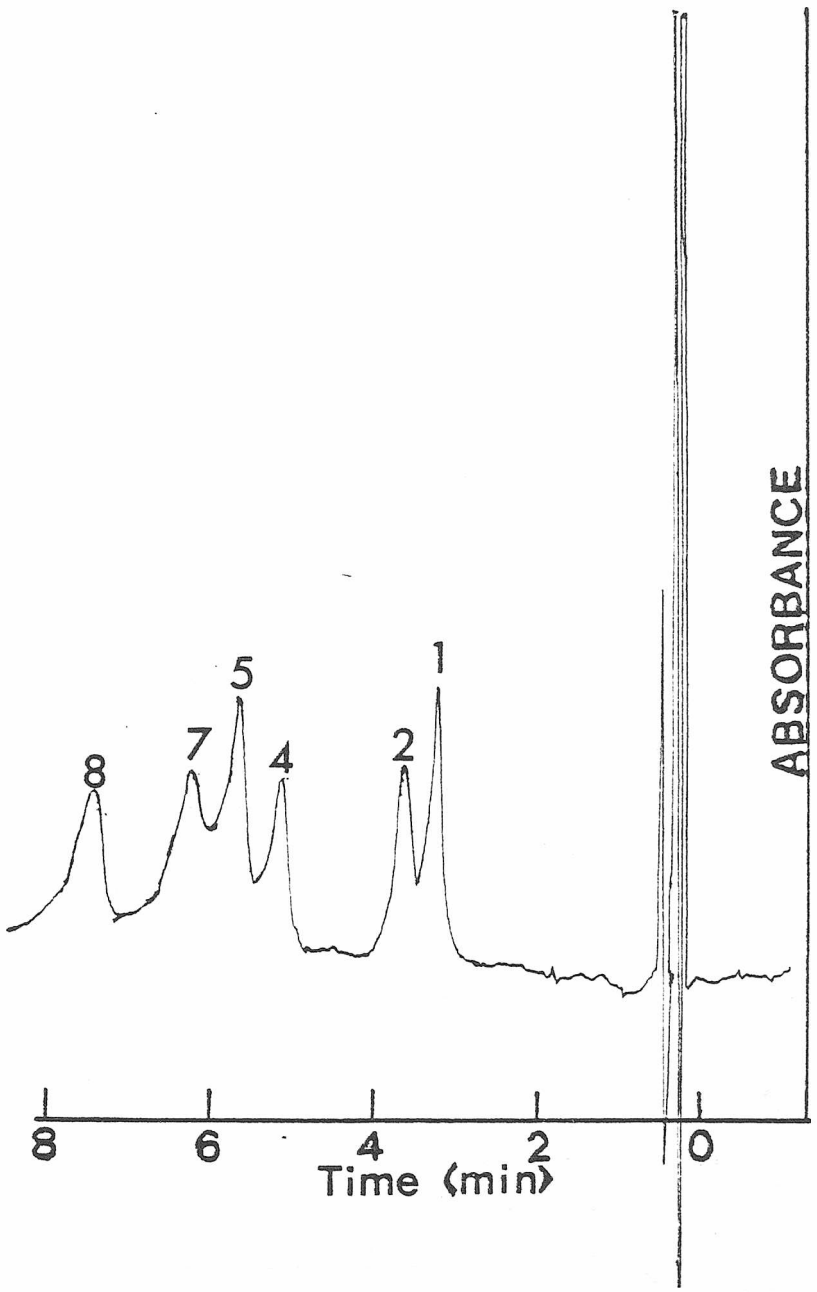


Figure 36 Chromatogram showing poor separation among certain tricyclic antidepressant drugs at high pH under ion-pairing condition. Chromatographic conditions as in Figure (35) with the pH of the eluent adjusted to 6.5. Compound identification as in Figure (35).

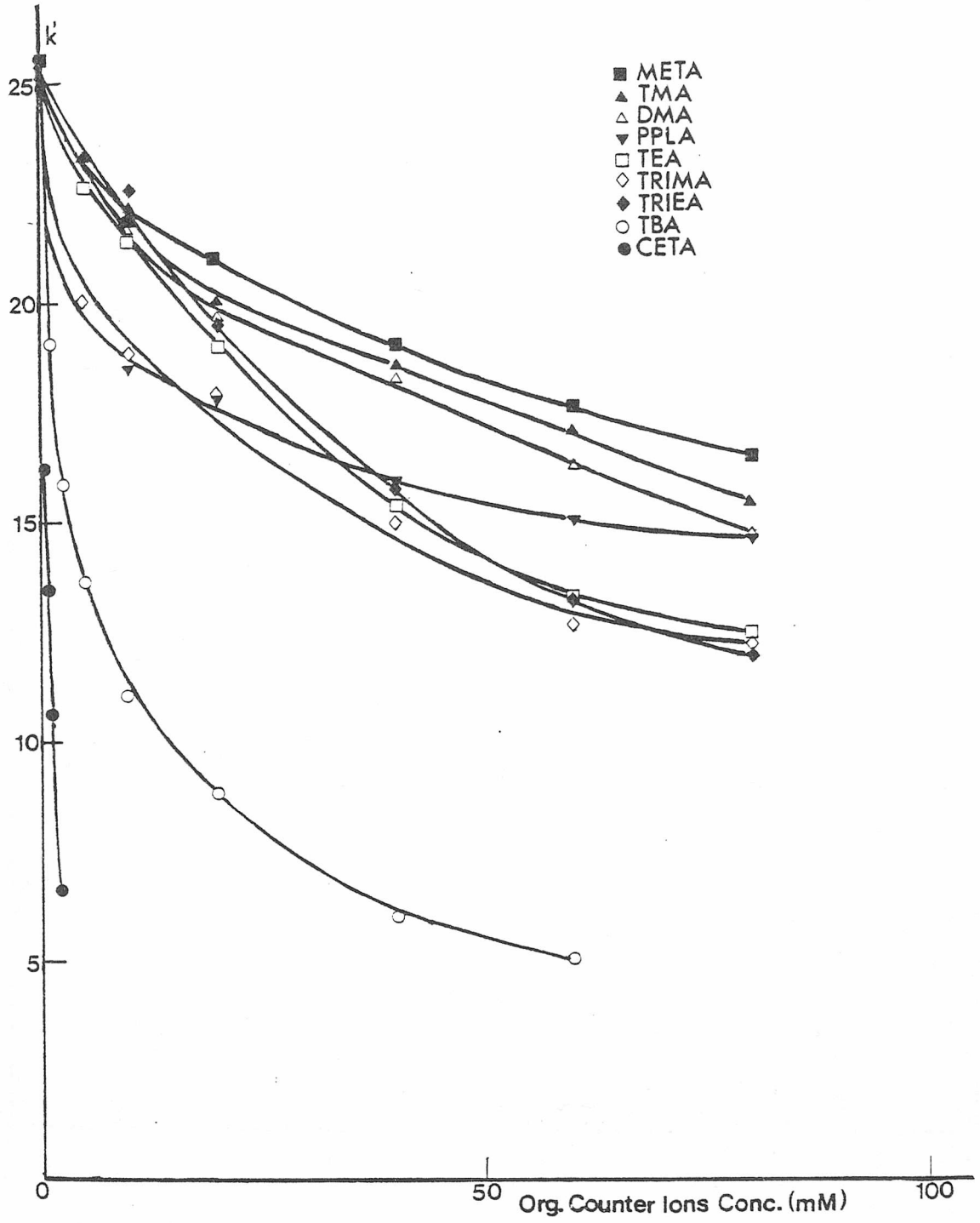


Figure 37 Plots showing the variation in capacity factor (k') for amitriptyline as a function of various added organic counter ion concentrations. Chromatographic conditions as Figure (35) using a 100 x 4.6 mm column.

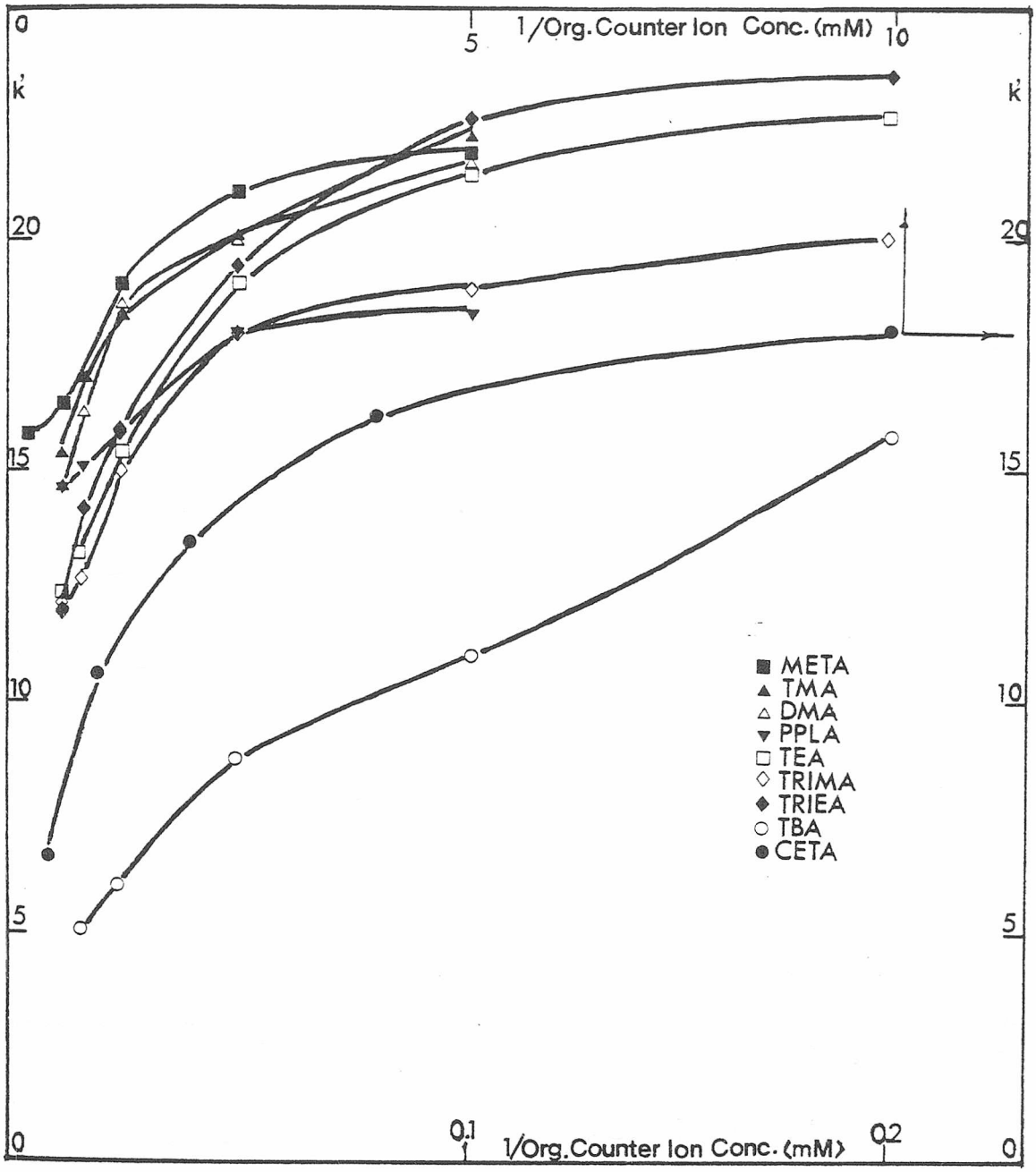


Figure 38a Plots showing the variation in capacity factor (k') for amitriptyline as a function of the reciprocal of the concentrations of various added organic counter ion. Chromatographic conditions as in Figure (37).

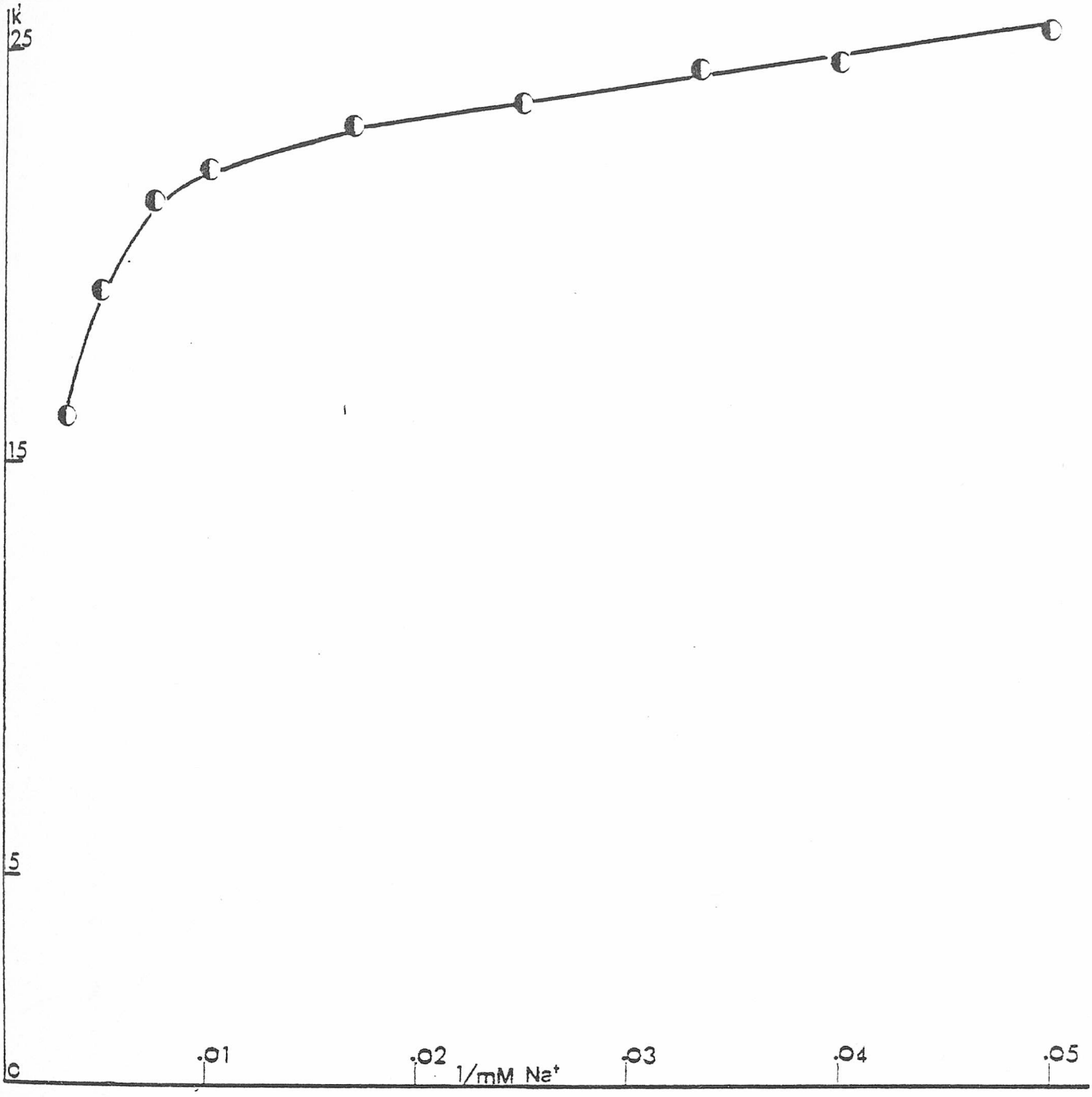


Figure 38b Plot showing the variation in capacity factor (k') of amitriptyline as a function of the reciprocal of the concentrations of added sodium ion. Chromatographic condition as in Figure (37).

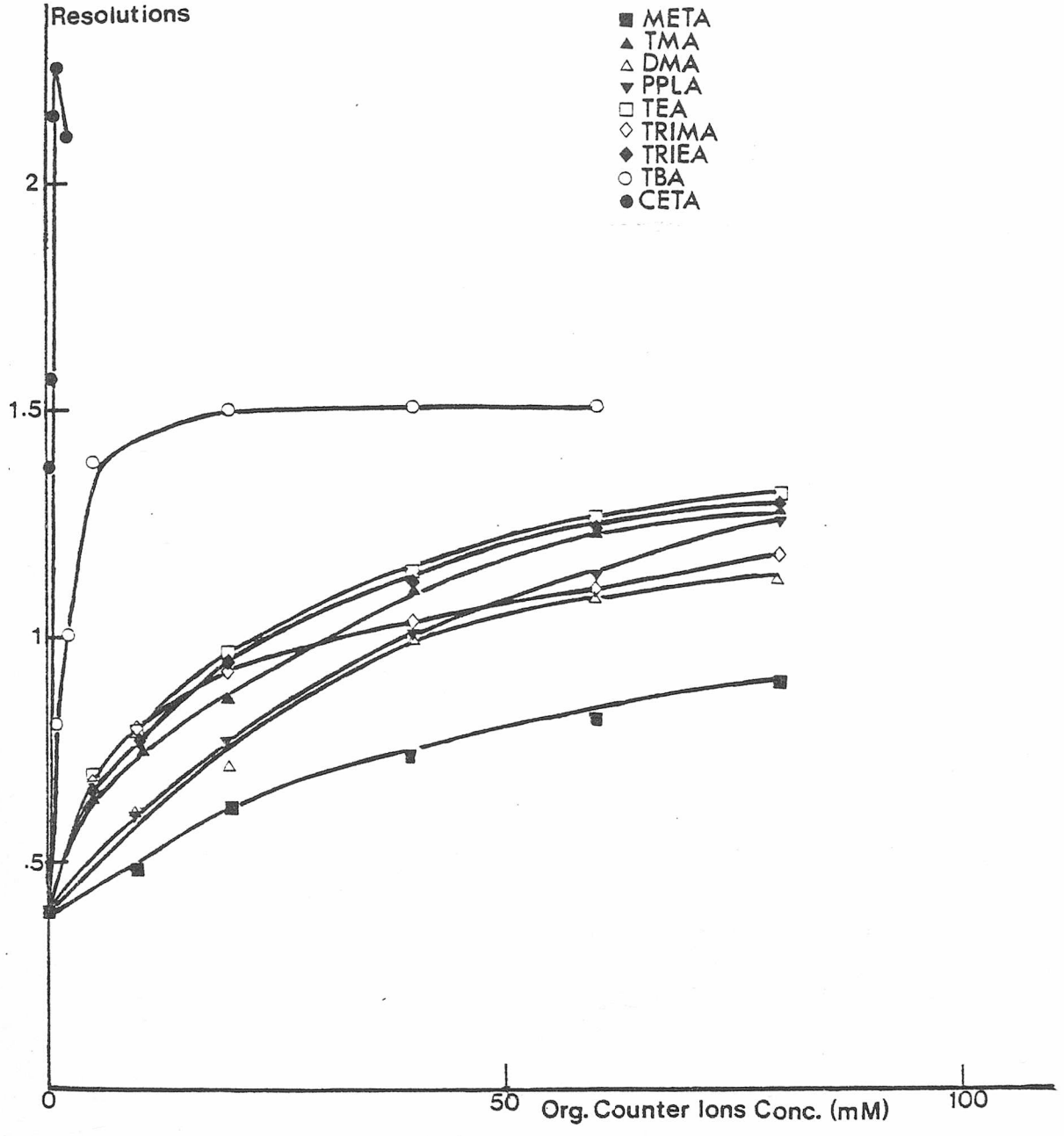


Figure 39 Plots showing resolution between amitriptyline and nortriptyline as a function of various added organic counter ion concentrations. Chromatographic conditions as in Figure (37).

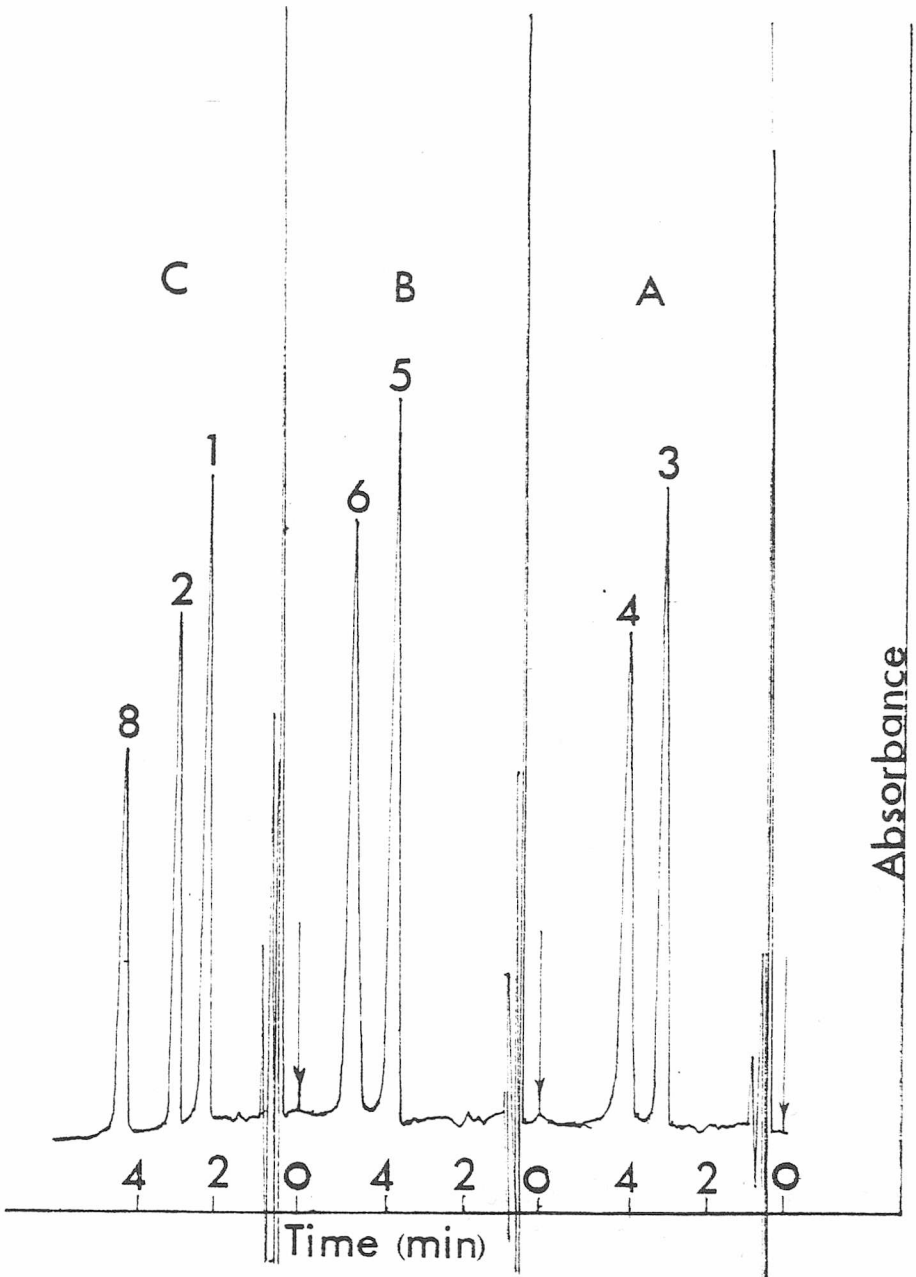


Figure 40 Representative chromatograms showing complete resolution obtained among the clinical important pairs amitriptyline and nortriptyline, imipramine and desipramine and among certain tricyclic antidepressants in presence of added organic counter ion (CETA). Chromatographic conditions as in Figure (35) with the addition of 2 mM (CETA) to the mobile phase using a 100 x 4.6 mm column. Compound identification as in Figure (35).

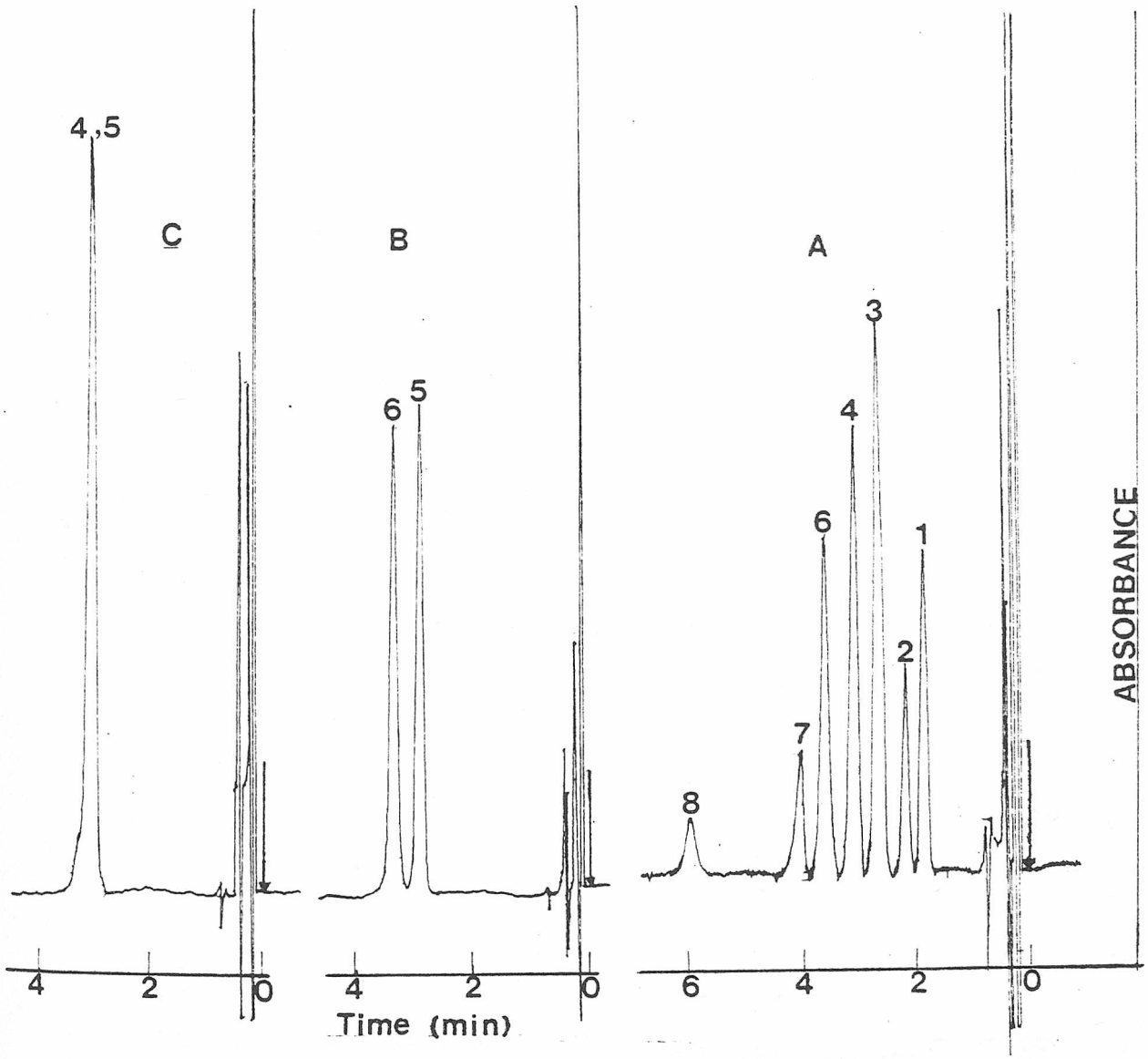


Figure 41 Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs in presence of added organic counter ion. Chromatographic conditions as in Figure (35) with the addition of 5 mM (TBA) to the mobile phase. Compound identification 1-6 as in Figure (35), 7 - maprotiline, 8 - nomifensine.

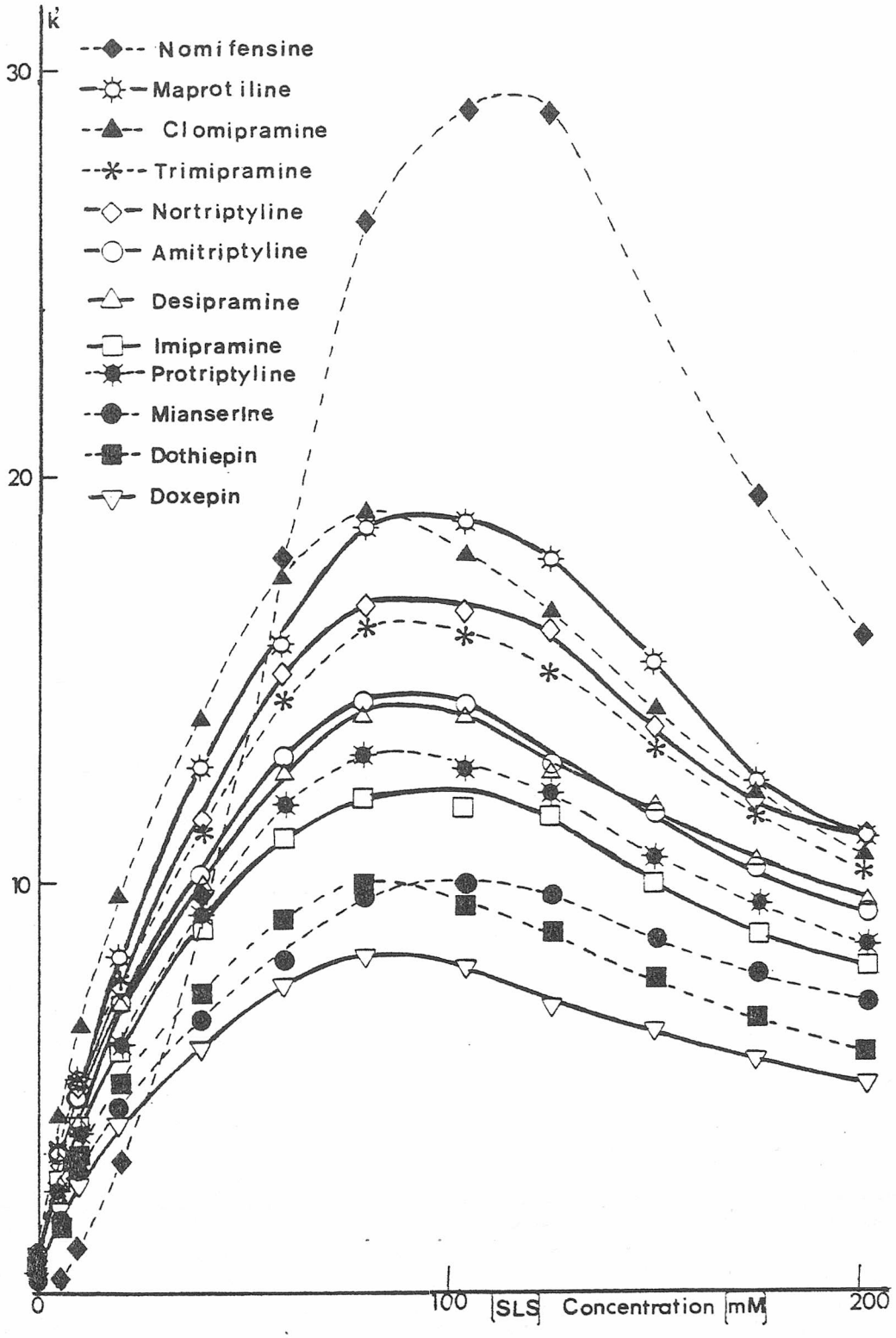


Figure 42 Plots showing variation in k' with mobile phase pairing ion (SLS) concentration for antidepressant drugs. Chromatographic conditions as in Figure (34) with the mobile phase modified to include 5 mM (TBA).

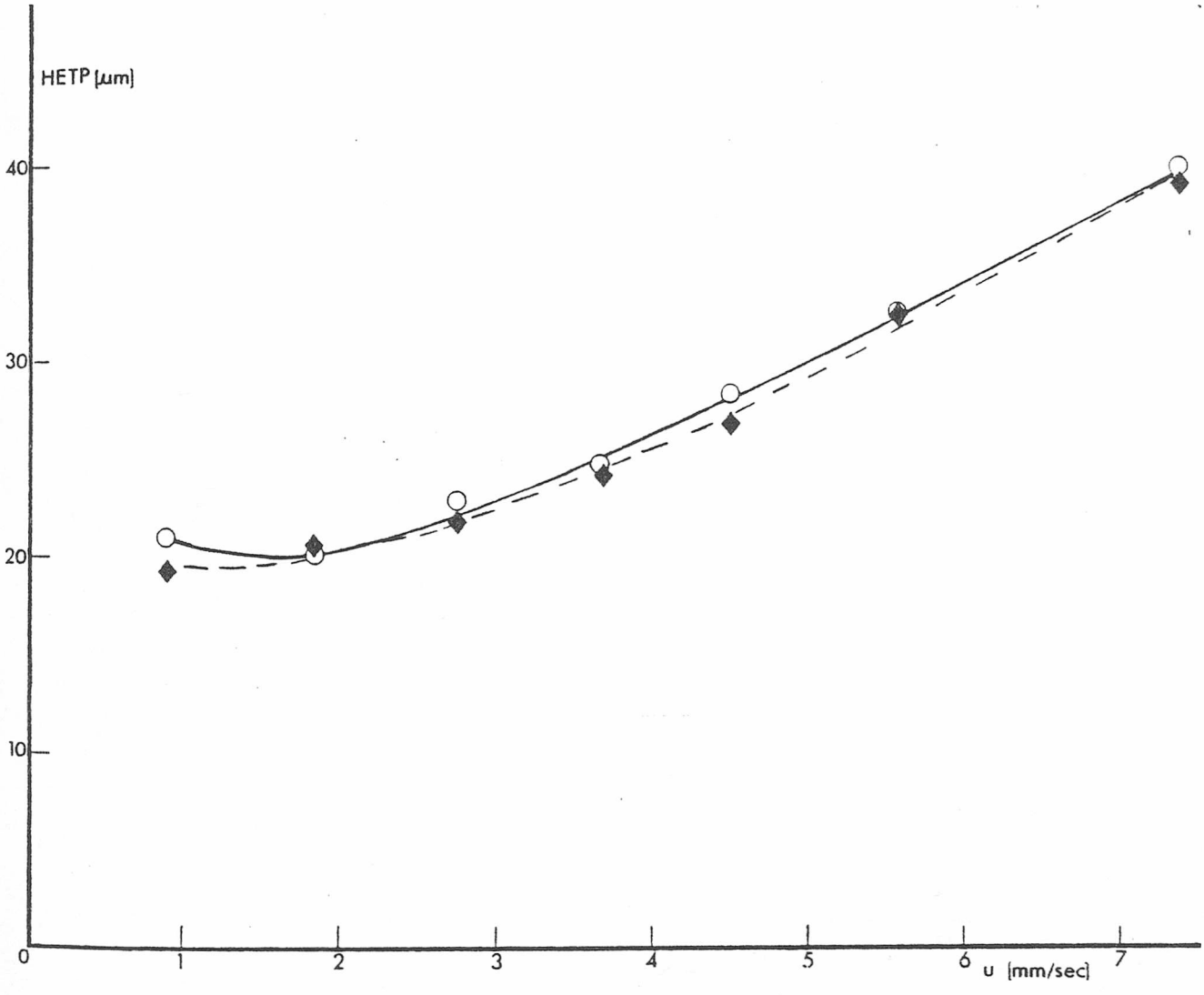


Figure 43 Plots showing variation of HETP with linear mobile phase velocity for amitriptyline. Chromatographic conditions: ——○——as in Figure (35), i.e. with no added counter ion, - - -◆- - - as in Figure (41), i.e. in presence of 5 mM (TBA).

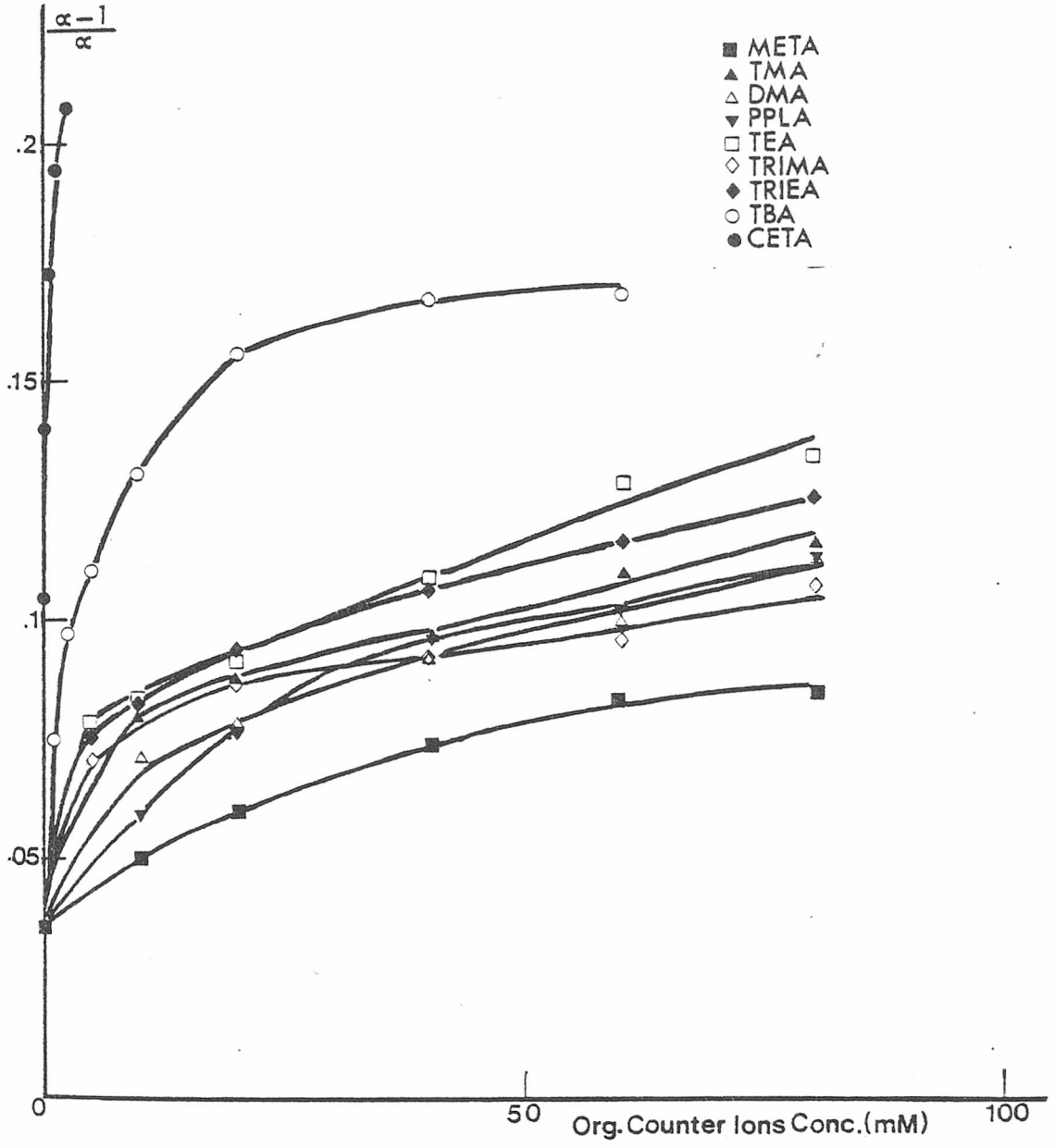


Figure 44 Plots of the variation of selectivity (α - 1)/α between amitriptyline and nortriptyline as a function of different organic counter ion concentrations. Chromatographic conditions as in Figure (37).

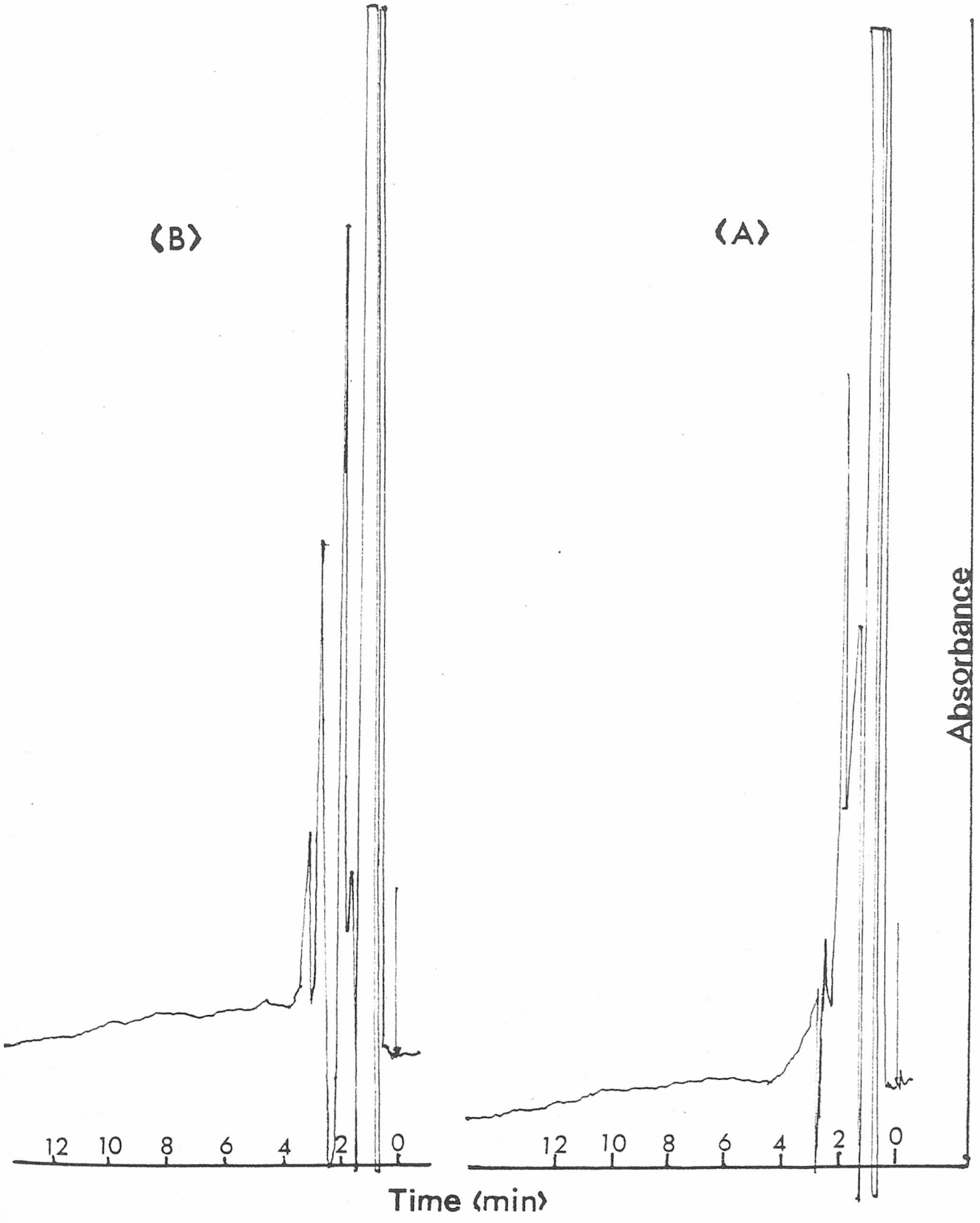


Figure 45 Chromatograms of serum blank extract; (A) wavelength of detection at 254 nm, (B) wavelength of detection at 230 nm. Full scale deflection of 0.005 absorbance units.

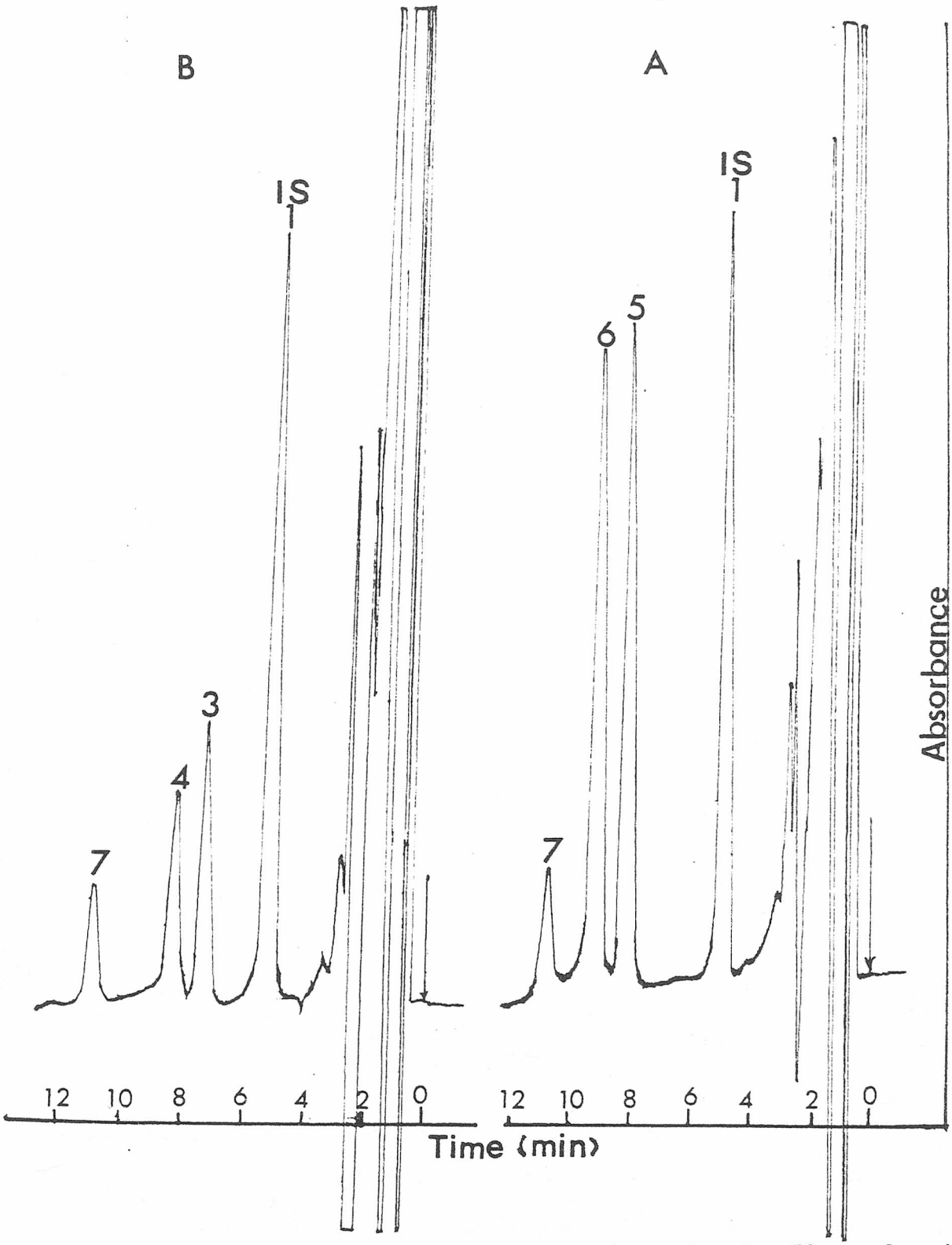


Figure 46 Chromatograms of serum extracts containing 50 ng of each of the tricyclic antidepressant drugs per cm^3 of serum. Chromatographic conditions and compounds identification are as in Figure (41) with wavelength of detection at 230 nm at full scale deflection of 0.005 absorbance units.

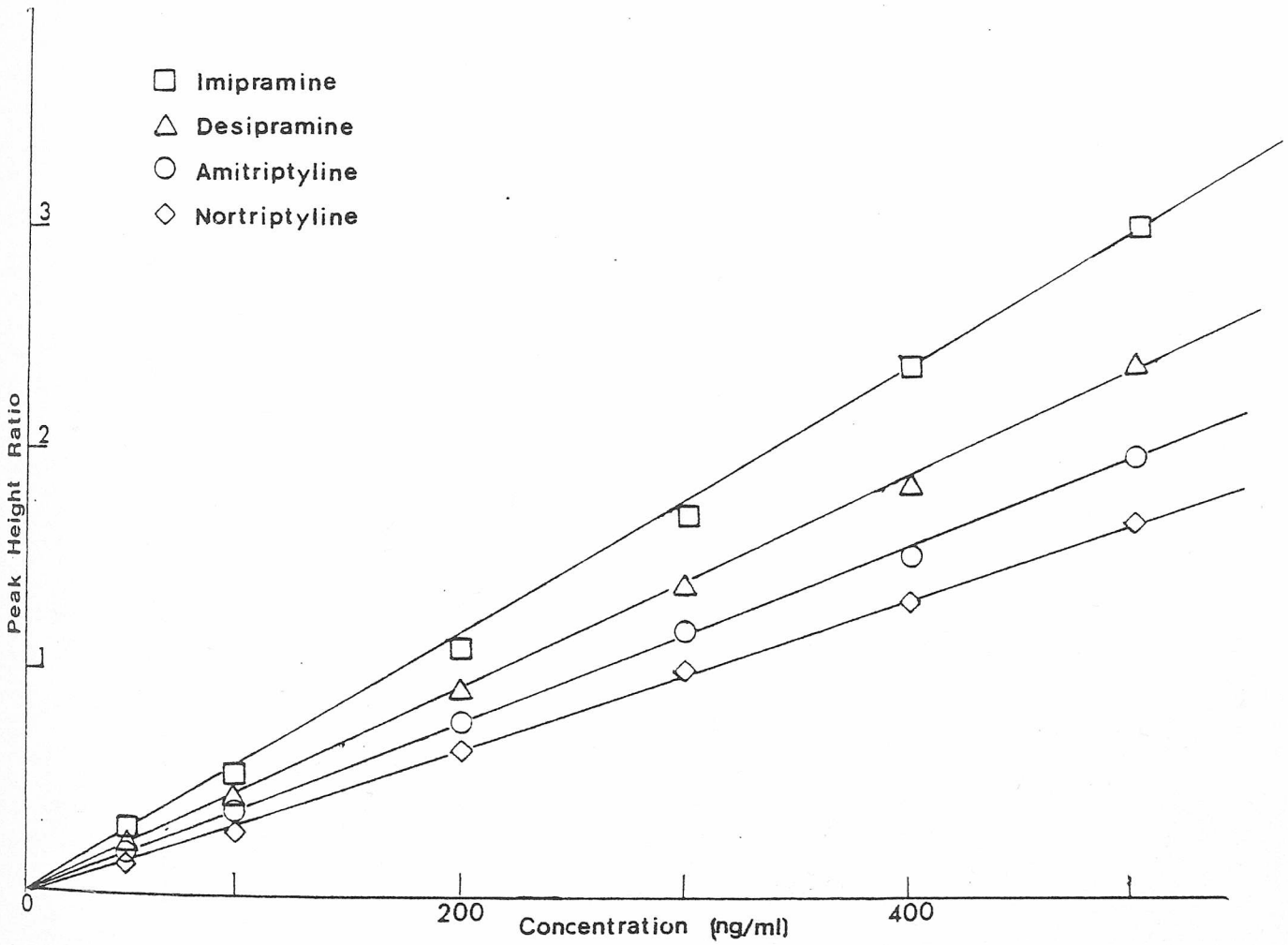


Figure 47 Calibration curves for the tricyclic antidepressants, peak height ratio of drug to internal standard vs. concentration for drug-free serum samples supplemented with amitriptyline, imipramine, desipramine and nortriptyline. Wavelength of detection at 254 nm. Internal standard doxepin was added to the sample at a concentration of 150 ng per cm^3.

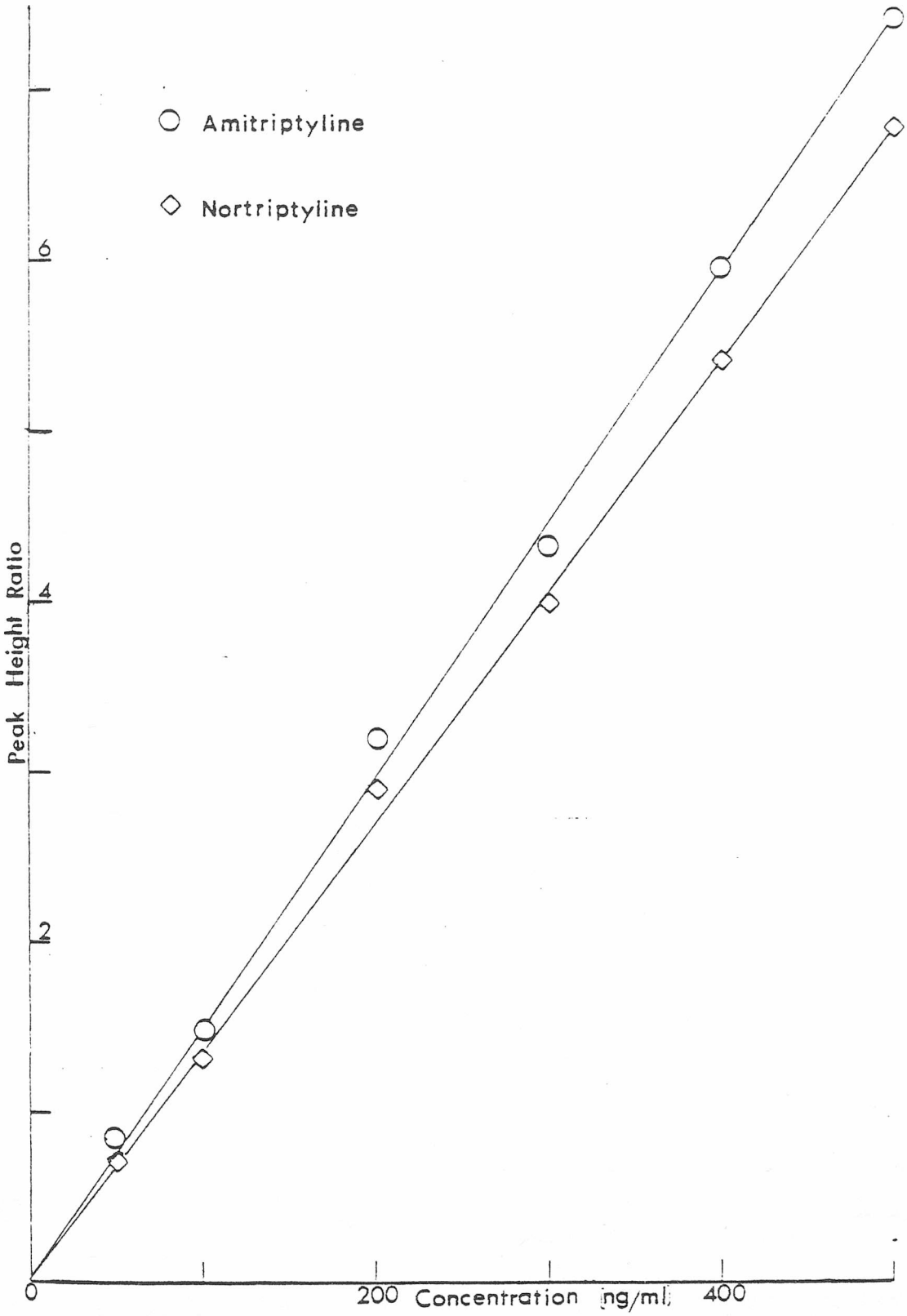


Figure 48a Calibration curves for the tricyclic antidepressants, peak height ratio of drug to internal standard vs. concentration for drug-free serum samples supplemented with amitriptyline and nortriptyline. Wavelength of detection at 230 nm. Internal standard doxepin was added to the sample at the concentration of 50 ng per cm^3.

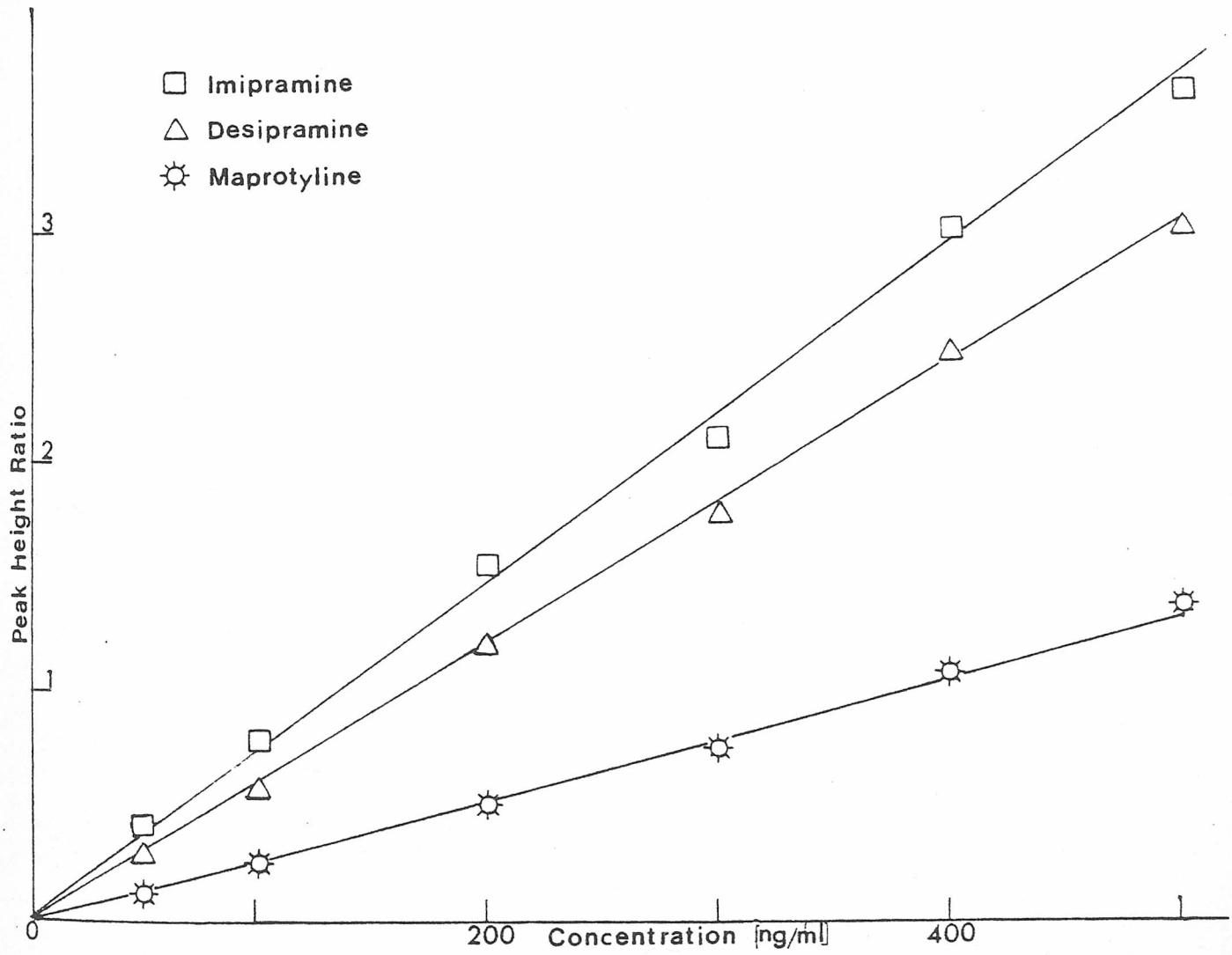


Figure 48b Calibration curves for the tricyclic antidepressants, peak height ratio of drug to internal standard vs. concentration for drug-free serum samples supplemented with imipramine, desipramine and maprotiline. Wavelength of detection at 230 nm. Internal standard doxepin was added to the sample at the concentration of 50 ng per cm^3.

μ cm sec^{-1}	Plate heights in μm					
	Nortriptyline		Imipramine		Desipramine	
	80 mM SLS	80 mM SLS + 5 mM TBA	80 mM SLS	80 mM SLS + 5 mM TBA	80 mM SLS	80 mM SLS + 5 mM TBA
0.0921	19.20	19.55	20.68	19.28	19.60	19.06
0.1842	21.24	22.24	21.42	21.77	20.86	19.59
0.2763	23.89	23.34	23.34	23.15	21.94	21.71
0.3684	25.39	26.84	26.34	26.81	24.14	25.08
0.4505	27.48	28.18	28.20	29.08	25.57	26.85
0.5526	32.66	34.66	30.67	32.65	31.31	33.22
0.7368	36.55	38.14	34.67	37.59	34.01	35.43

Table (6)

Table showing the values of plate height for nortriptyline, imipramine and desipramine as a function of mobile linear velocity. Chromatographic conditions as in Figure (35).

Compound	SLS (80 mM)			SLS (80 mM) + TBA (5 mM)		
	$K'_{des}{}^{K}_{IE}$	$K'_{des}{}^{K}_{IE}$ Ratio	α	$K'_{des}{}^{K}_{IE}$	$K'_{des}{}^{K}_{IE}$ Ratio	α
Imipramine	0.179	1.04	1.05	0.0955	1.188	1.129
Desipramine	0.185			0.114		
Amitriptyline	0.208	1.06	1.04	0.109	1.250	1.123
Nortriptyline	0.219			0.136		
Desipramine	0.185	1.12	1.13	0.114	1.05	1.03
Amitriptyline	0.208			0.109		

Table (7)

Table showing the observed separation factors and the calculated ratio of ion exchange desolvation constants $K'_{des}{}^{K}_{IE}$ derived from Equation 4 in presence and absence of organic counter ion for selected pairs of compounds.

Drug	Slope	Y-Intercept	R
Amitriptyline	3.936×10^{-3}	$+ 2.440 \times 10^{-3}$	0.9998
Nortriptyline	3.413×10^{-3}	$- 1.628 \times 10^{-3}$	0.9998
Imipramine	6.065×10^{-3}	$- 4.136 \times 10^{-2}$	0.9986
Desipramine	4.765×10^{-3}	$- 2.397 \times 10^{-2}$	0.9994

Table (8)

Table showing the linear regression data for the calibration curves of the tricyclic drugs with the wavelength of detection at 254 nm using internal standard doxepin at 150 ng ml^{-1} of serum.

Drug	Slope	Y-Intercept	R
Amitriptyline	1.474×10^{-2}	$+ 4.764 \times 10^{-2}$	0.9991
Nortriptyline	1.343×10^{-2}	$+ 5.821 \times 10^{-2}$	0.9990
Imipramine	7.232×10^{-3}	$+ 3.653 \times 10^{-2}$	0.9987
Desipramine	6.113×10^{-3}	$- 1.815 \times 10^{-2}$	0.9995
Maprotiline	2.752×10^{-3}	$- 2.987 \times 10^{-2}$	0.9979

Table (9)

Table showing the linear regression data for the calibration curves of the tricyclic drugs with the wavelength of detection at 230 nm using internal standard doxepin at 50 ng ml^{-1} of serum.

Drug	Within Day			Day-to-Day		
	n	Mean	CV%	n	Mean	CV%
50 ng ml^{-1}						
Amitriptyline	6	54.10	3.9	9	52.08	6.8
Nortriptyline	6	51.75	3.6	9	49.25	5.4
Imipramine	4	47.58	5.3	8	50.12	6.3
Desipramine	4	46.72	5.4	8	47.28	7.2
250 ng ml^{-1}						
Amitriptyline	5	253.12	1.9	10	251.50	4.5
Nortriptyline	5	248.31	2.6	10	249.32	5.6
Imipramine	4	247.26	3.3	8	250.53	5.6
Desipramine	4	255.30	3.5	8	256.41	5.2

Table (10)

Table showing the precision of assay for the tricyclic antidepressants in serum with wavelength of detection at 254 nm.

Drug	Within Day			Day-to-Day		
	n	Mean	CV%	n	Mean	CV%
50 ng ml^{-1}						
Amitriptyline	5	53.69	5.9	10	51.81	9.2
Nortriptyline	5	52.54	6.8	10	49.32	10.3
Imipramine	4	51.93	3.2	9	50.90	6.2
Desipramine	4	46.89	4.5	9	47.24	6.5
Maprotiline	4	47.21	6.2	9	46.83	8.8
250 ng ml^{-1}						
Amitriptyline	4	242.58	6.7	12	254.36	8.2
Nortriptyline	4	251.60	6.9	12	252.47	8.4
Imipramine	5	253.75	4.5	8	250.74	6.3
Desipramine	5	257.68	2.6	8	256.53	7.2
Maprotiline	5	250.56	7.3	8	248.95	9.9

Table (11)

Table showing the precision of assay for the tricyclic antidepressants in serum with wavelength of detection of 230 nm.

Sample Code No.	Amitriptyline			Nortriptyline			Imipramine			Desipramine		
	S	M	T	S	M	T	S	M	T	S	M	T
1279	389	225	364	15.8	175	0	150	156.3	165.2	200	226.5	250.7
280	–	–	–	–	–	–	202	181	201.2	136.8	108.6	146.6
380	283	257.2	234	48.2	49.5	45	–	–	–	–	–	–
880	–	173.6	213	–	149.2	136	–	–	–	–	–	–
1080	249.2	233.2	226	188.2	217.3	166	99.6	105.1	106.3	351.2	387.6	344.5
1180	–	–	–	–	–	–	439	388	446	293	294.7	316
181	107.5	89.1	93	163.7	156.7	124	–	–	–	–	–	–
281	–	–	–	–	–	–	61.6	50.4	70.3	195.8	168.4	230.1
481	43.2	38	59	89.7	89.2	80	243	190	191.6	57.4	45.8	72.9

Table (12)

Table showing the comparison of the results between those obtained by different laboratories using different analytical techniques and that obtained in the present investigation.

S – is the spike serum concentration of the drug in ng ml^{-1}.

M – is the mean serum concentration of the drug in ng ml^{-1} tested by different laboratories using different analytical techniques.

T – is the serum concentration of the drug obtained by this investigation.

Drug	Recovery %			
	Concentration in ng ml^{-1}			
	50	100	200	400
Doxepin	72.6	75.1	84.9	90.8
Desipramine	63	68.3	71.2	88
Imipramine	69.6	73.3	74.4	75.4
Amitriptyline	68.4	73.8	84	81.3
Nortriptyline	63	62.5	66	68.4
Maprotiline	67.1	70.9	72.8	82

Table (13)

Table showing the mean percentage recovery of the tricyclic drugs from serum (n = 4).

CHAPTER FIVE

THE USE OF REVERSED PHASE ION-PAIR HPLC IN THE SEPARATION AND DETERMINATION OF PROGUANIL AND ITS METABOLITES

5.1 Introduction

Proguanil hydrochloride (Paludrine, ICI) was synthesised by Curd et al. in 1945 [232] and is still the chief agent employed in malaria therapy. It exerts causal prophylactic and suppressive activity in sporazite-induced falciparum malaria, adequately controls the acute clinical attack and usually eradicates the infection.

Proguanil is rather slowly but adequately absorbed from the gastro-intestinal tract and is very rapidly eliminated from the tissues after its administration ceases. It has been reported by Smith et al. [233] that, in man, 40-60% of the proguanil absorbed is excreted in urine and 10% secreted directly into the intestine and eliminated in the feces as the parent compound. 30% of the drug is partially metabolised, by cyclisation in the liver, to give the metabolite cycloguanil. Subsequent metabolism results in the formation of another metabolite 4-chlorophenylbiguanide. The structure of proguanil and its suggested metabolites are shown in Figure (49).

It has been reported that, the metabolite cycloguanil is the actual compound possessing the prophylactic activity rather than the parent drug proguanil [234]. On the other hand, it has been demonstrated by several workers that it is far more effective to administer proguanil rather than the so-called active metabolite cycloguanil against the malarial infection both in rhesus monkeys [235] and in humans [236].

Recently by use of reversed phase ion-pair HPLC, with ODS-Hypersil as the stationary phase and sodium lauryl sulphate as the pairing ion in a mobile phase of 50% v/v acetonitrile buffer, Taylor et al. [123] were able to separate and to determine proguanil and its metabolites in serum at a level of 60 ng ml^{-1}. Measurement of the drug concentration in the serum of a human volunteer after ingestion of 200 mg of proguanil over a period of 24 hours showed that neither the active metabolite cycloguanil nor the

non-active one 4-chlorophenylbiguanide was observed.

Such an observation may lead to the suggestion that, it is the proguanil itself which posses the antimalarial activity. However, in the above method the retention of the cycloguanil was inadequate, because the drug was not well resolved from endogenous serum materials. Furthermore, the serum drug concentration was determined after deproteination with perchloric acid, and it has been demonstrated recently that such a step may cause up to 50% losses of drug [237]. It appears that refinement of this analytical method will be required if the presumably lower levels of the suggested metabolites are to be measured.

5.2 Scope of investigation

With the information on retention of ionic solutes in ion-pair chromatography obtained in Chapter (2) available, it was intended in this study to improve the resolution of the cycloguanil from the endogenous serum constituents. At the same time, it was intended to keep the retention of the drugs 4-chrlorophenylbiguanide and proguanil within acceptable time limits.

In view of the reported drug losses in the deproteination step before chromatography of the serum sample, it was intended to improve the detection limit by use of liquid extration methods as discussed in Chapter (4). Also, the use of liquid extraction of a drug from serum, can produce a more concentrated sample and a much cleaner solution.

Furthermore, it has been reported by Taylor et al. [123] that the retention times of the proguanil and its metabolite have a linear dependence on the mobile phase pairing ion concentration and such an observation is at variance with that predicted by the ion-exchange-desolvation mechanism. Thus, it was also the aim of this investigation to study the retention behaviour of these biguanide bases under ion-pair conditions using a much

wider range of mobile phase pairing ion concentration in order to observe the predicted maxima and verify further the generality of the behaviour described in Chapter (2).

5.3 Experimental

Apparatus

The liquid chromatograph used consisted of an Altex (Model 110A) constant flow pump. Detection at 254 nm was by a Cecil (CE 2012) variable wavelength detector. Injection was by a Rheodyne Model 7120 valve fitted with 20 or 100 µl loops. Columns were 100 mm long and 4.6 mm internal diameter, slurry packed with 5 µm ODS-Hypersil (Shandon, London, Great Britain) as described in Section (2.3.3).

Materials

Proguanil, cycloguanil and 4-chlorophenylbiguanide were kindly donated by ICI (Macclesfield, Great Britain). Pairing ion, sodium lauryl sulphate (SLS), and buffer salt disodium hydrogen phosphate dodecahydrate were obtained from Fisons Ltd. (Leicister, Great Britain). The extraction solvents, n-hexane and isoamyl alcohol were purchased from BDH (Poole, Great Britain). Tetrabutylammonium bromide was obtained from Aldrich Chemical (Milwaukee, U.S.A.) and acetonitrile was obtained from Rathburn (Walkerburn, Great Britain). Water was glass distilled and all other chemicals used were of AnalaR grade.

Extraction procedure

All glassware was silanised prior to use with a 2% v/v solution of dimethylchlorosilane in 1,1,1-trichloroethane.

To 2 ml of serum sample in a 10 ml conical centrifuge tube 500 µl of 1 N sodium hydroxide and 5 ml of hexane/isoamyl alcohol mixture (4:1) were added and shaken gently to avoid emulsion formation for 3 minutes.

The tube was centrifuged for 3 minutes at 3000 rpm. The organic layer was then transferred to another centrifuge tube and the above extraction procedure was repeated with a further 4 ml of the extraction solvent. To approximately 9 ml of organic solvent recovered 100 µl of 0.5 M sulfuric acid was added and the mixture shaken vigorously for 3 minutes. After centrifuging for 3 minutes at 3000 rpm, the organic solvent was discarded and the aqueous extract injected for chromatography.

5.4 Results and discussion

From the results shown in previous chapters it can be seen that in ion pairing chromatography there are several variables peculiar to this technique which can be adjusted to modify the retention time and capacity factor of an ionic solute. How they are used, will depend upon the purpose to which the chromatography is to be put. In the case of the tricyclic antidepressants the main objective was selectivity coupled with sensitivity. In the present study on the antimalarials, selectivity is not so important and the purpose is more to reduce the retention time of proguanil in order to increase the detection limit and to increase that of cycloguanil so that it can be detected independently from endogenous materials.

The variables to be considered in selecting a chromatographic system while being interrelated e.g. the organic modifier concentration will alter pairing ion adsorption, can be listed as follows:

1. Organic modifier concentration: This will in general have the effect that increase in organic modifier concentration will produce shorter retention times with consequent loss of separation but increase in sensitivity of detection.

2. pH: This, as has been outlined in Chapters (3) and (4) can markedly affect retention by changing the dissociation state of a solute.

In ion pairing chromatography it is general practice to ensure that the solute is fully ionised in order to utilise retention process based on electrostatic interactions.

3. Pairing ion concentration in the mobile phase: While this has been taken as the relevant parameter by many workers, it would appear from the results in Chapter (2) that the important quantity is the adsorbed surface pairing ion concentration. Although this is controlled primarily by the nature of the pairing ion and by its mobile phase concentration, it is also markedly affected by the organic modifier concentration in that, as has been demonstrated in Chapter (2), the isotherm will be considerably changed by change in the organic content of the mobile phase. The effect of adsorbed pairing ion is complex as has been demonstrated for both acidic and basic solutes in Chapter (2). That is, at low loadings the effect of increasing pairing ion concentration is to increase retention, but when the surface is saturated with respect to a particular pairing ion, increase in pairing ion concentration will have the opposite effect of reducing retention. In addition the concentration ranges at which these effects become apparent will depend markedly on the hydrophobicity of the pairing ion.

4. Counter ion concentration: Retention can be altered according to classical ion exchange ideas by utilising the reciprocal relationship between capacity factor and counter ion concentration. That is, an increase in counter ion concentration will produce a decrease in k'. In Chapter (4) it was indicated that organic species, used as counter ions also decreased the capacity factors but did not obey the reciprocal relationship even at very low concentrations. Their inclusion did however modify retention of the tricyclics to different extents and thus alter the basic selectivity of the overall chromatographic system.

5. Buffer concentration: In ion pairing systems the effect of inorganic buffer salts will be two fold,

(a) they may act as counter ions as outlined above and reduce retention as their concentration increases,

(b) they also act to fix the state of ionisation of the solutes.

In system containing no organic counter ion, the buffer salts fulfil both of these functions. When organic counter ions are included, as in the case of tricyclic drugs separation as shown in Chapter (4), it would appear that buffer salts function only to control ionisation. The much greater effect of organic species means that they dominate as counter ions.

In order to optimise the separation of proguanil and its metabolites the above variables were examined independently, although it is realised that the effects observed would be altered in magnitude by the inclusion of the pairing ion in solution. The effect of pH, however will be quite different in presence of pairing ion and the purpose of preliminary measurements in absence of pairing ion serves to establish the regions of pH where complete dissocation occurs.

5.4.1. Effect of the acetonitrile content

The results of examining the effect of organic modifier, acetonitrile on the retention of the three protonated bases are shown in Figure (50). At low concentrations i.e. below 40% v/v, the retention times, in terms of the capacity factors, are seen to depend very strongly on organic modifier concentration. Above this value the capacity factors are largely independent of acetonitrile. Thus, use of organic modifier to control retention will result in a very critical system which would result in low reproducibility in terms of retention unless concentrations of acetonitrile above 40% v/v are used. 50% v/v acetonitrile was chosen as the organic modifier content. While, the retention will be markedly altered by the

addition of pairing ion, the effect of organic modifier in ion pairing systems will be similar and the very marked increase in stability of the chromatographic system at high organic modifier concentration overides any advantages to be gained by achieving retention on the basis of reduced acetonitrile concentration. In addition the chromatograms obtained in presence of low acetonitrile concentrations showed very broad peaks presumably as a consequence of poor mass transfer under ion-exchange conditions. Specimen chromatograms are shown in Figure (51A-B).

5.4.2 Effect of pH

It has been shown in the last chapter that pH of the eluent will affect the retention of an ionic solute in ion-pair chromatography. Figure (52) demonstrates the effect of altering pH of the eluent on the capacity factors of the biguanides. It is seen that k' values increase as the pH of the eluent increases. At the same time peak broadening becomes apparent, especially in the case of proguanil, at above pH 4 the peak shape is distorted due to severe peak tailing as shown in Figure (53). The pH of the eluent was thus fixed at pH 2, since at this pH the peak shape is sharp. Moreover, at this pH, the k' of the solutes is insensitive to the changes of hydrogen ion concentration. Also at this pH all the bases are fully protonated and can therefore be expected to have their retention enchanced by addition of pairing ion.

5.4.3 Effect of the buffer salt concentration

As with the tricyclic antidepressant drugs, it was observed that addition of the buffer salt causes a marked decrease in the retention of cycloguanil, 4-chlorophenylbiguanide and proguanil as is shown in Figure (54). At above 10 mM disodium hydrogen phosphate concentration, further increases in the salt concentration cause little reduction in the retention of the solutes. The buffer salt concentration in the eluent was therefore fixed

at 10 mM. As in the case of the tricyclic drugs, no explanation can be given to account for the observed reduction of the k' of the solutes on addition of inorganic salt to the eluent.

5.4.4 Effect of the mobile phase pairing ion concentration

In view of the 50% v/v acetonitrile content of the mobile phase, the use of a pairing ion of low hydrophobicity e.g. (PANS) will be less effective as a means of increasing the retention of the solutes than a very hydrophobic pairing ion, such as SLS. It has been demonstrated in Chapter (2) that SLS at 50% v/v acetonitrile will still be appreciably adsorbed on to the C-18 surface and will thus function effectively as a pairing ion in increasing k' values.

The variation of the k' of cycloguanil, 4-chlorophenylbiguanide and proguanil as a function of mobile phase sodium lauryl sulphate concentration is demonstrated in Figure (55). Figure (55) shows that the capacity factors of the three biguanide derivatives go through the usual maxima and that the maximum k' is achieved at a SLS mobile phase concentration of 90 mM. The elution order between the cycloguanil and 4-chlorophenylbiguanide is seen to have altered at 8 mM mobile phase SLS. Such a change in selectivity on variation of pairing ion concentration has been demonstrated in the last two chapters and also has been reported by several other workers [123,153].

Although maximum separation among these three biguanides can be obtained at 90 mM mobile phase SLS concentration, the k' of the proguanil at this concentration is very large. This, besides being time consuming for routine analysis, also reduces the detection limit of the system for proguanil. However, retention of these solutes at 200 mM SLS eluent concentration is within acceptable limits, where the k' of cycloguanil is 6 and that of proguanil is 19. Moreover, excellent separation was

observed among these three solutes at 200 mM SLS. The chromatogram of these solutes at 200 mM SLS in 50% v/v acetonitrile buffer is demonstrated in Figure (56). It can be observed from Figure (55) that, although at above 90 mM mobile phase SLS concentration, further addition of the pairing ion into the eluent causes a general decrease of the k' of the solutes, the degree of decrease of the retention among these solutes is different. The retention of proguanil is seen to be drastically reduced as the SLS concentration increases after the maximum, while cycloguanil is observed to be very insensitive to increase in the mobile phase pairing ion concentration.

According to the ion-exchange-desolvation mechanism, the decrease in the k' of solutes on increase of mobile phase pairing ion concentration after the maximum is due to the decrease in area available for desolvation of the solute and the increase in mobile phase counter ion concentration. It has been demonstrated in the last chapter that addition of small concentrations of organic counter ion into the eluent in ion-pair chromatography can cause marked decrease in k' of the solutes. It was thus thought that, the required retention and separation of these biguanides could be achieved by the addition of a suitable amount of organic counter ion rather than by use of such extremely high pairing ion concentrations. While lower concentrations of SLS could be employed i.e. below the maximum to achieve separation, this could result in a situation where retention was very sensitive to SLS concentration and in much greater resolution between proguanil and 4-chlorophenylbiguanide than is required. An alternative approach might be to increase the acetonitrile concentration at a SLS concentration of 90 mM i.e. the maximum. This however would yield unacceptably small values of k' for cycloguanil.

5.4.5 Influence of the organic counter ion

It has been demonstrated in the last chapter that TBA is an effective

counter ion in reducing the retention in ion-pair chromatography of the tricyclic antidepressant drugs. Thus TBA was used as the organic counter ion to adjust the k' of the three biguanides in this study. Use of organic counter ion in the present investigation was to alter the k' of the solutes to acceptable time limits, especially proguanil, rather than to change the selectivity of the stationary phase as was in the case for the tricyclic antidepressants.

The variation of k' of the solutes as a function of added TBA in an eluent containing 90 mM SLS in 50% v/v acetonitrile buffer is shown in Figure (57). The k' values of all three biguanide derivatives are seen to decrease as the mobile phase TBA concentration increases. This effect indicates the generality of the observations recorded in Chapter (4) for the tricyclics. Again, the relationship between capacity factor and reciprocal TBA concentration is seen not to be linear as is shown in Figure (58).

In Figure (57), it can be observed that, 5 mM TBA in a mobile phase of 90 mM SLS in 50% acetonitrile buffer can provide a retention pattern for the three solutes very similar to that obtained in Figure (56), where 200 mM SLS is used. A chromatogram of these solutes in the eluent including TBA is shown in Figure (59). The retention data of the biguanides in presence of 5mM TBA as a function of mobile phase SLS concentration are shown in Figure (60).

From Figure (60), it can be observed that, in presence of 5mM TBA, the k' of all solutes is markedly decreased. However, the maximum in k' of all solutes with pairing ion concentration is still apparent and is again obtained at 90 mM SLS. Figure (60) also indicates that, at 90 mM SLS concentration, slight variation of the pairing ion concentration will cause minimal alteration of the retention values of the solutes.

The eluent containing the lower concentration of pairing ion and

including the small concentration of TBA is preferable to that involving the very high concentration of pairing ion concentration alone. Firstly, it is more economic. Also it minimises refractive index changes observed when low ionic strength samples are injected into chromatographic solvents having high concentrations of pairing ions especially those having surface active properties such as cetrimide and SLS. In addition it was found experimentally that very high concentration of SLS were very difficult to degas before chromatography.

It was decided on the basis of the above experimental findings that the most satisfactory eluent for the analysis of the three biguanides under investigation was, a 50% v/v acetonitrile–buffer, 10 mM disodium hydrogen phosphate, 90 mM in SLS and 5mM in TBA at a pH of 2.0.

5.4.6 Extraction of the drugs from serum

It was found that, all glassware used for the extraction of the biguanide derivatives had to be silanised before use. It was found that up to 25% of these compounds could be lost during extraction when untreated glassware was used.

Ether and hexane are probably the most popular solvents used for extraction of lipophilic drugs from aqueous systems. This is because they are less dense than water, so that they can easily be removed by use of suction after extraction. Also emulsion formation does not occur with either of these solvents upon shaking during the extraction procedure. Furthermore liquid extraction with the use of hexane or ether as the organic solvent can usually provide a reasonably clean extract.

Thus, hexane and ether were initially employed in the extraction of the biguanides derivatives from human serum. Both of these solvents were found to give satisfactory recovery of the drugs 4–chlorophenylbiguanide and proguanil from the basified serum. However, neither hexane nor ether was able to extract cycloguanil.

This suggests that a strong hydration shell is formed around the cycloguanil, so that is will not partition into the hexane nor into the ether even in its neutral state. The chemical structure of cycloguanil indicates that it is a strong electron donating compound which will theoretically dissolve in any solvent with sufficient electron accepting property. The obvious organic solvents having this property are the higher alcohols, where the hydroxy group of the alcohol will interact with the electron lone pair of the nitrogen atoms of the cycloguanil through hydrogen bonding.

Hexane–isoamyl alcohol mixture was thus used as the organic solvent in the extraction of the biguanide derivative from serum in this investigation. It was discovered that high isoamyl alcohol content in the extraction solvent would cause emulsion formation upon shaking during extraction. At the same time the aqueous acidic back extract from the organic hexane–isoamyl alcohol solvent contained large amounts of endogenous serum constituents that would interfere with the cycloguanil peak in chromatography. Insufficient isoamyl alcohol content, on the other hand, would cause poor recovery of the cycloguanil. Experiments indicated that hexane–isoamyl alcohol in the ratio 4:1 would provide satisfactory recovery of drugs consistent with a reasonably clean extract. A chromatogram of a blank serum extract using the above extraction solvent is shown in Figure (61A). Most of the endogenous UV–absorbing serum constituents eluted at retention times less than 3 minutes and did not interfere with the analysis. A chromatogram of an extract from serum containing 25 ng ml^{-1} of cycloguanil, 4–chlorophenylbiguanide and proguanil is shown in Figure (61B).

The percentage of recoveries of these drugs were also calculated as in Chapter (4). Aliquots of drug free serum were supplemented with the three drugs to give concentrations of 50 and 250 ng ml^{-1}. The results

listed in Table (14) represent the average of four extraction of each of the two spiked serum pools. The recoveries ranged around 80% and extraction efficiencies were comparable for the three biguanides at each concentration.

5.4.7 Quantitation

The calibration curves of peak height, at full scale absorbance of 0.01, against concentration of drugs in serum were found to be linear as shown in Figure (62). The constants of the respective linear regression lines are listed in Table (15).

No internal standard is included in the above method. Preliminary investigation showed that it was difficult to obtain a compound with similar extraction properties which could be clearly separated from the three biguanides. Before the above method could be used as an effective monitoring system for the drugs in serum such a suitable internal standard would be required. It is suggested, however, that the chromatography of these three bases is critical and that the above system represents an advance upon the previous work involving these compounds [123].

5.5 Conclusion

It has been demonstrated for three biguanide bases that many of the chromatographic variables found to be important in the case of the tricyclic antidepressant drugs are equally applicable to this, quite different, set of compounds. The parabolic relationship between k' and mobile phase pairing ion concentration is again confirmed. This fact enables a suitable pairing ion concentration to be selected which optimises the separation among the solutes and also uses a system which is not critical with respect to pairing ion or indeed any other chromatographic variable.

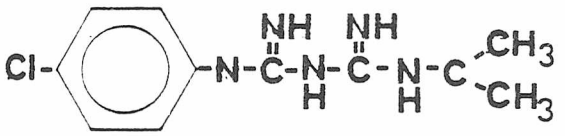
This solvent requires a very high pairing ion concentration with

its consequent practical difficulties. The parabolic relationship, taken in conjunction with the general behaviour of compounds used as organic counter ions, enables an alternative eluent to be predicted. This solvent with lower pairing ion concentration and including a small concentration of organic counter ion produces equally good chromatography in terms of separation of the bases and reasonable retention times both for proguanil and cycloguanil.

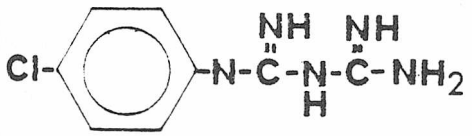
It is concluded from the results obtained in Chapters (4) and (5) that the effects of the variables outlined in Section (5.4) are reasonably accurate and general. Consideration of these variable has, in the case of the biguanides, provided a very adequate separation for the purposes of quantitative assay and this approach, in principle, should be very generally applicable to other classes of compounds.

It is shown that the extraction procedure required for concentration of these compounds is very critical especially in the case of cycloguanil and a solvent system of hexane–isoamyl alcohol is suggested as being adequate in terms of recovery and reproducibility. A detection limit of 5 ng ml^{-1} using a 2 ml serum sample is obtainable taking cycloguanil as the most difficult compound.

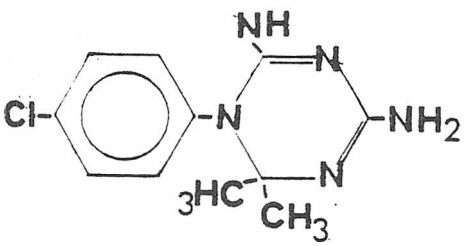
No suitable internal standard, however, has been found and before this method could be reliably used an appropriate internal standard to monitor recoveries from the extraction procedure would be required.



Proguanil



4-Chlorophenylbiguanide



Cycloguanil

Figure 49 Chemical structures of proguanil and its suggested metabolites 4-chlorophenylbiguanide and cycloguanil.

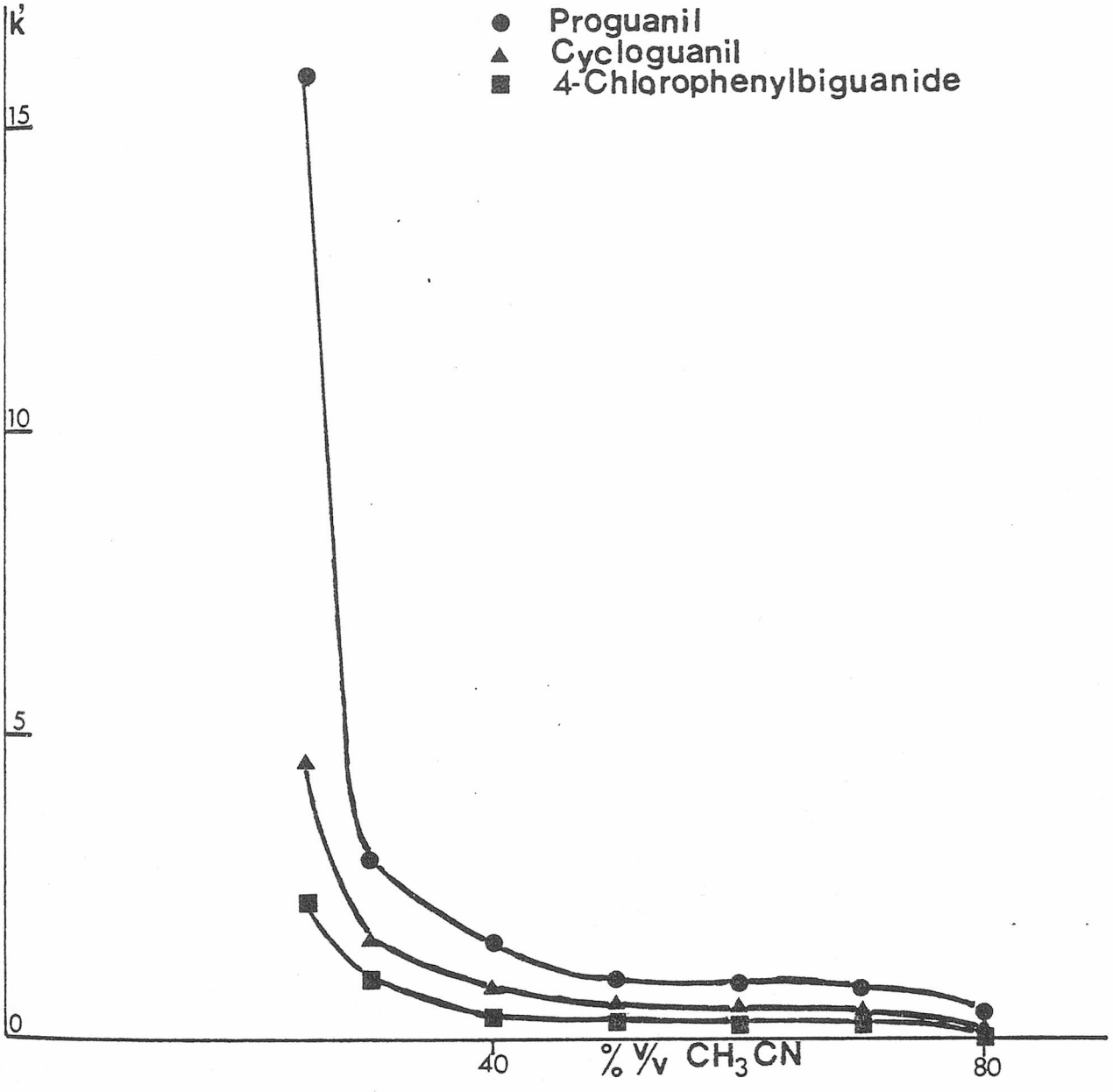


Figure 50 Influence of the acetonitrile content of the eluent on capacity factor (k') of the three biguanides. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil column; mobile phase acetonitrile-buffer of 10_3mM disodium hydrogen phosphate at pH 2; flow rate at 2.0 cm^3 min^{-1}.

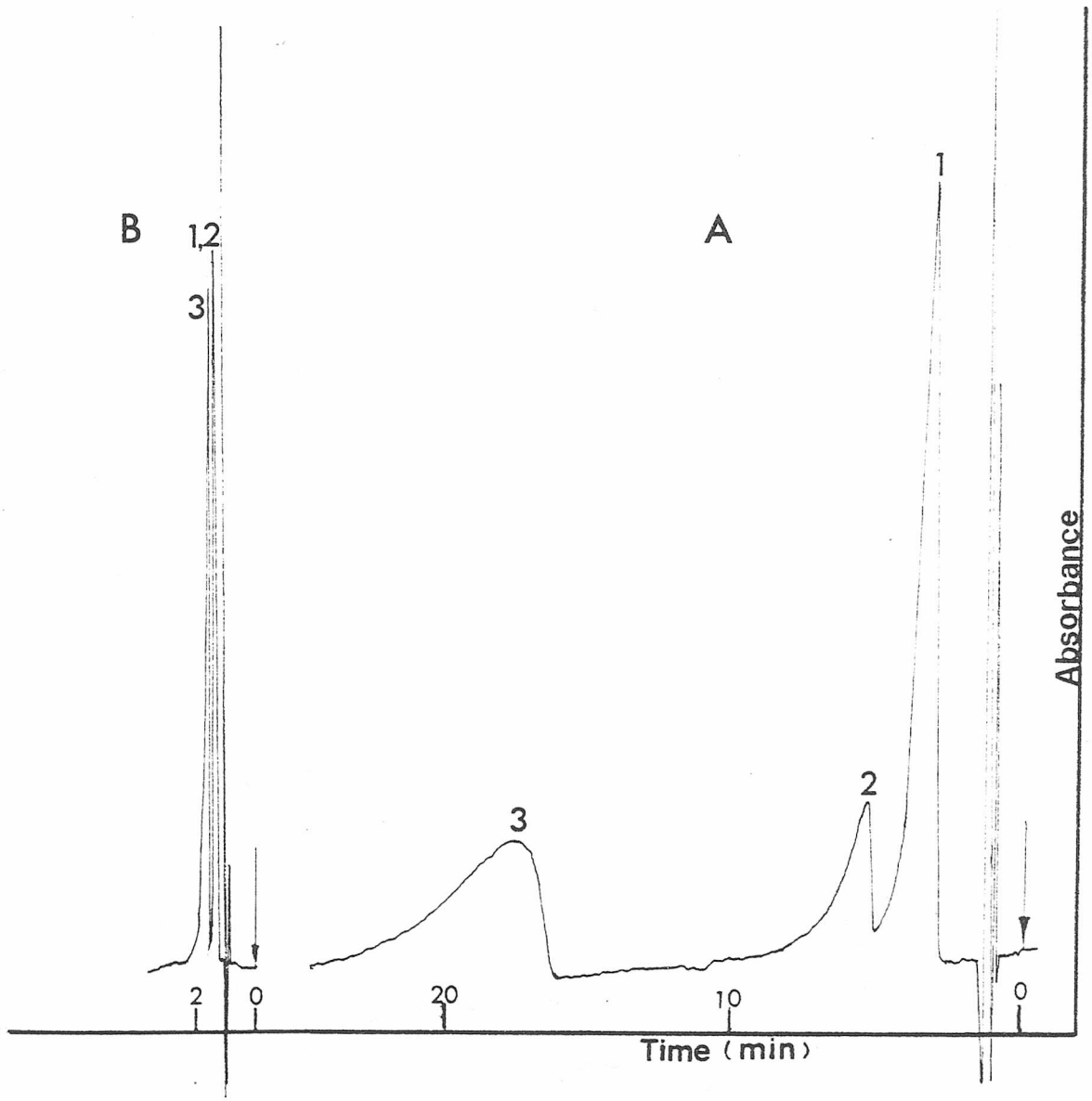


Figure 51 Chromatograms showing the influence of acetonitrile content on the retention and separation of proguanil and its metabolites;

(A) solvent 25% v/v acetonitrile buffer at pH 2,
(B) solvent 50% v/v acetonitrile buffer at pH 2.

Column used were 100 x 4.6 mm ODS-Hypersil.
Compounds identification: 1 - 4-chlorophenylbiguanide; 2 - cycloguanil; 3 - proguanil.

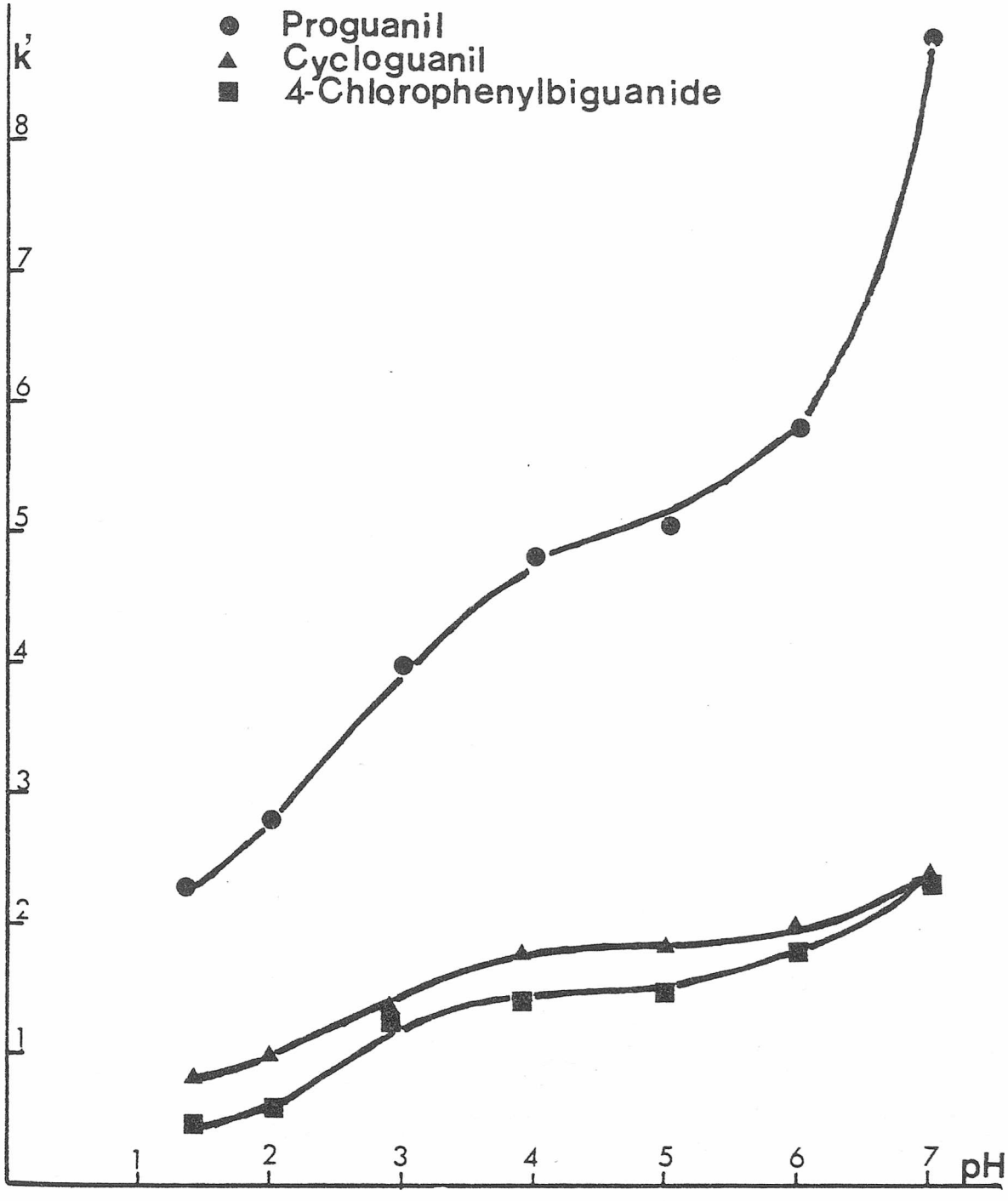


Figure 52 Plots showing the effect of pH on capacity factor (k') of the three biguanides. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil column at 2.0 cm^3 min^{-1} flow rate; mobile phase of 50% v/v acetonitrile-buffer of 10 mM disodium hydrogen phosphate.

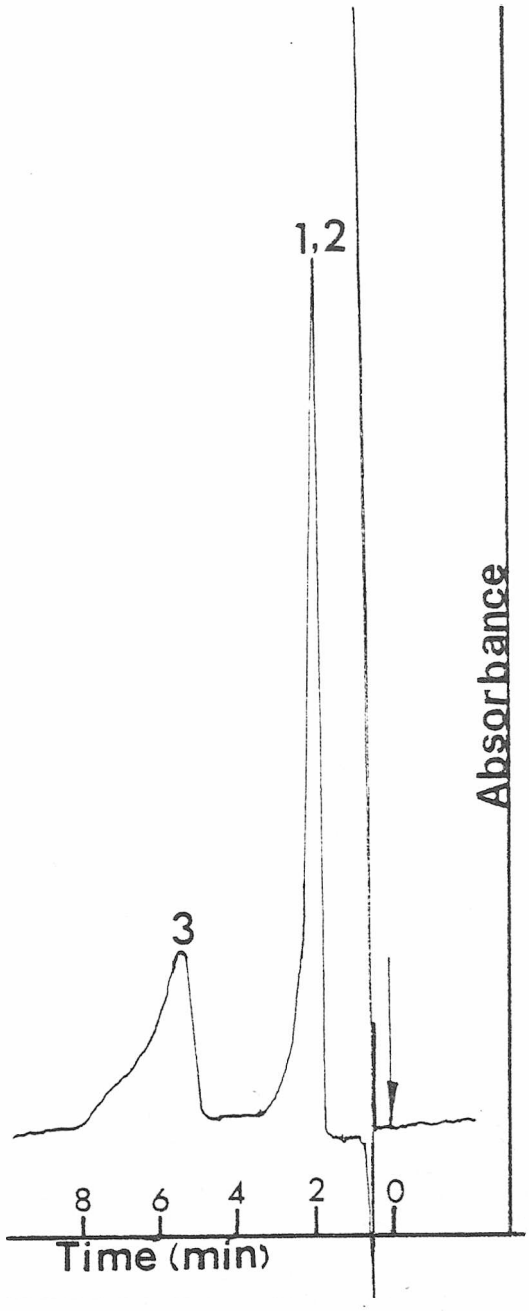


Figure 53 Chromatogram showing the retention and separation of the three biguanides at high pH mobile phase. Chromatographic conditions as in Figure (52) with pH of the eluent adjusted to 7. Compounds identification as in Figure (51).

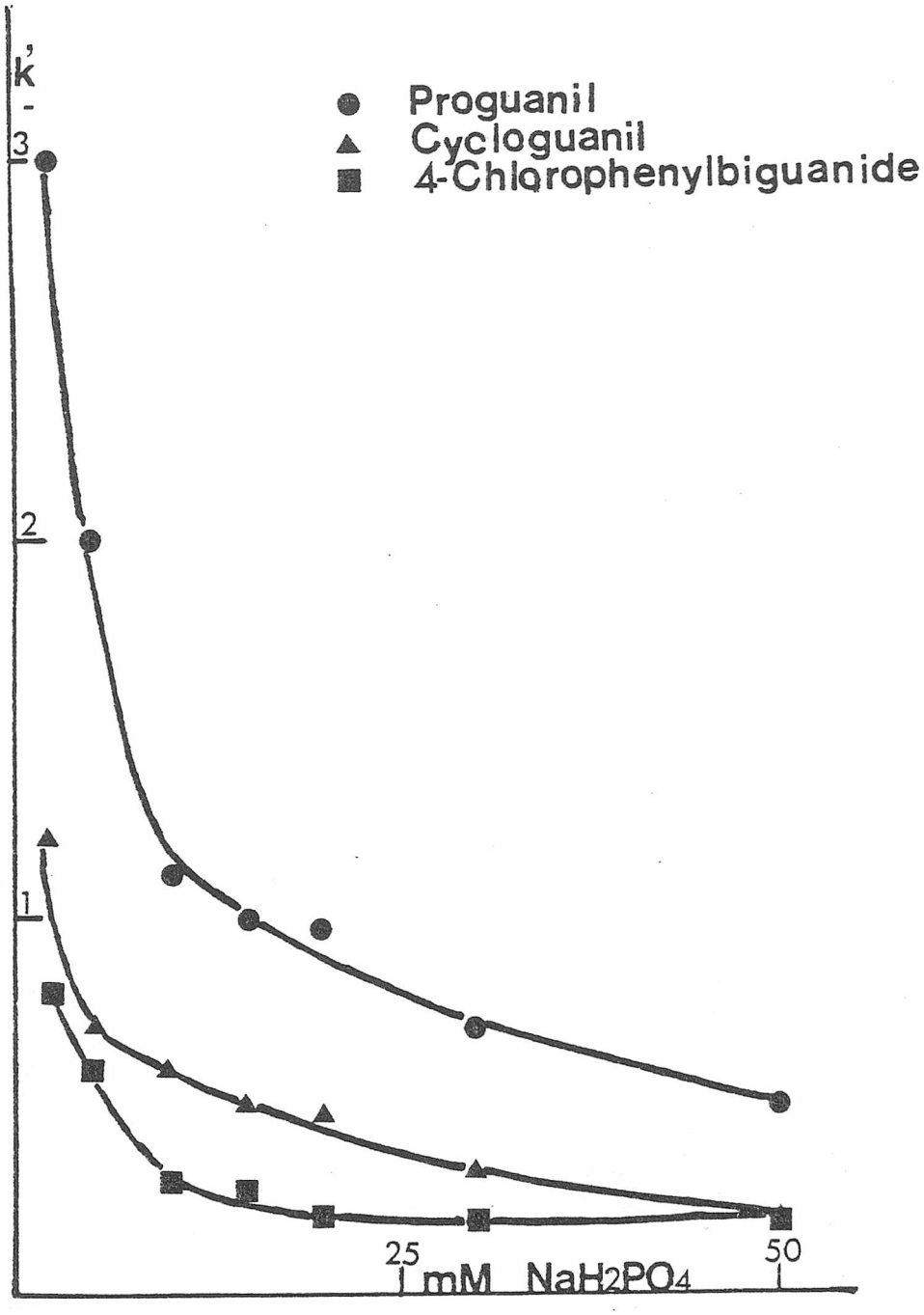


Figure 54 Influence of the buffer salt disodium hydrogen phosphate concentration on capacity factor (k') of the three biguanides. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil, solvent 50% v/v acetonitrile-buffer at pH 2, flow rate 2.0 cm^3 min^{-1}.

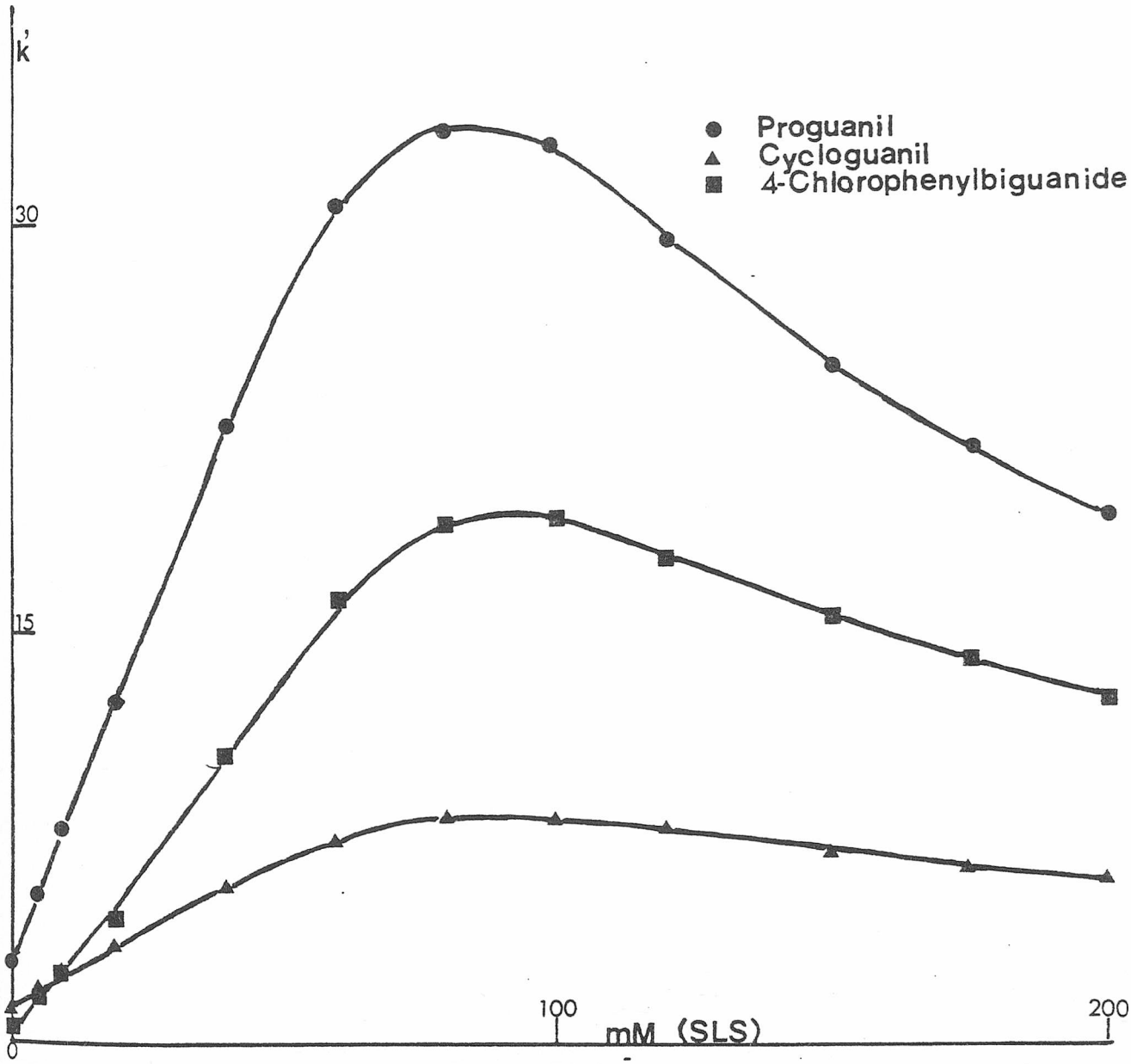


Figure 55 Plots showing the variation of capacity factor (k') with mobile phase pairing ion (SLS) concentration for the three biguanides. Chromatographic conditions: 100 x 4.6 mm ODS-Hypersil column; mobile phase 50% v/v acetonitrile-buffer of 10 mM disodium hydrogen phosphate at pH2. Flow rate at 2 cm^3 min^{-1}.

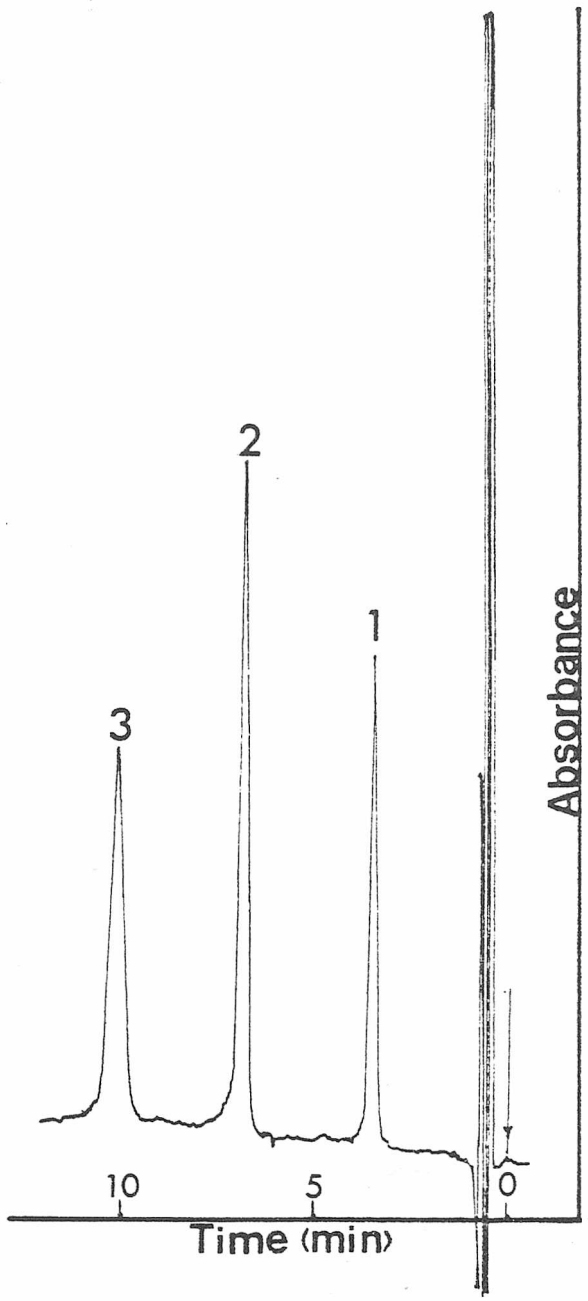


Figure 56 Chromatogram showing the complete separation of the three biguanides using extremely high mobile phase pairing ion (SLS) concentration. Chromatographic conditions as in Figure (55) with the addition of 200 mM (SLS) into the eluent. Compounds identification as in Figure (51).

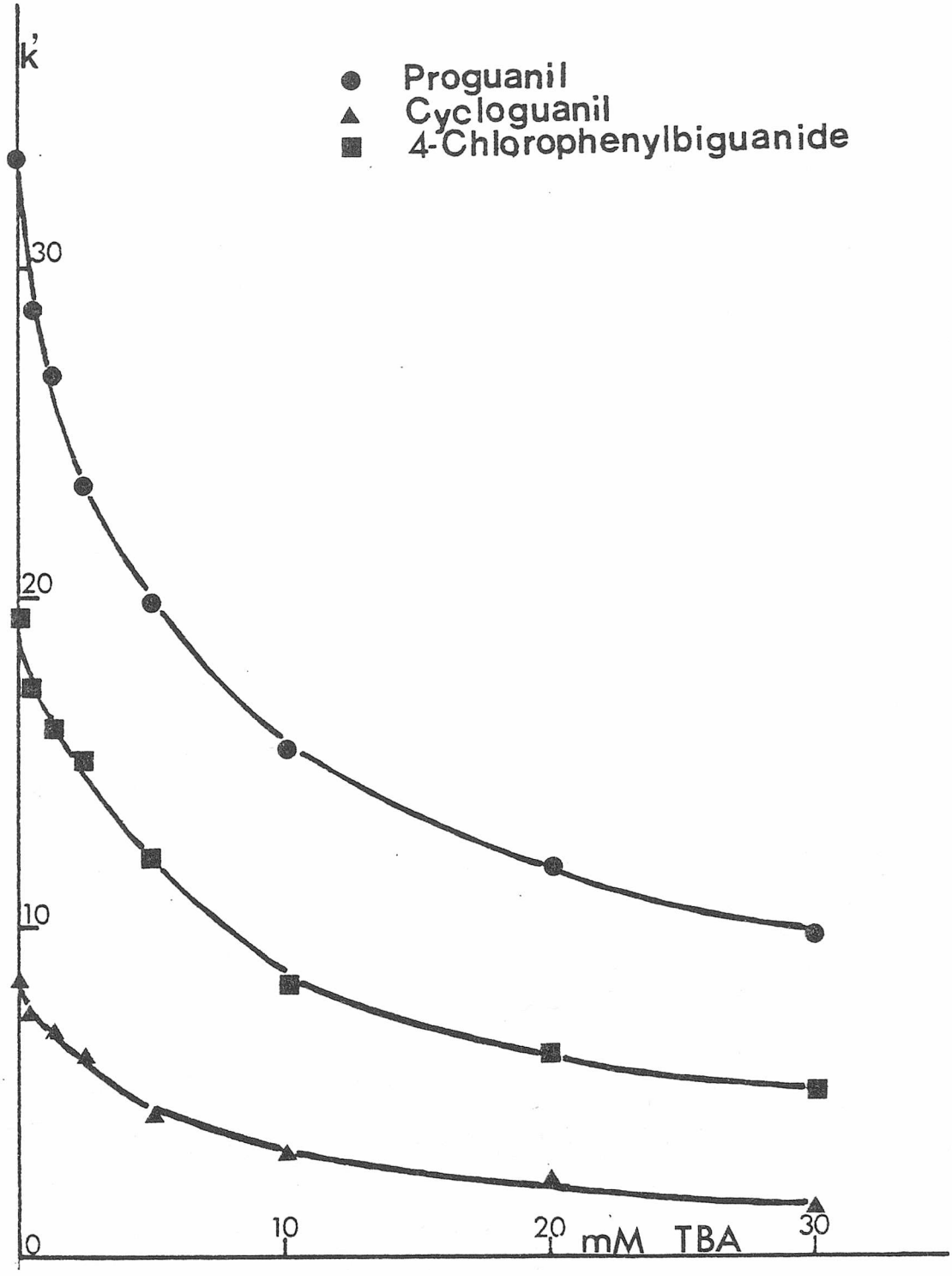


Figure 57 Plots showing the variation in capacity factor (k') for the three biguanides as a function of organic counter ion (TBA) concentration. Chromatographic conditions as in Figure (55).

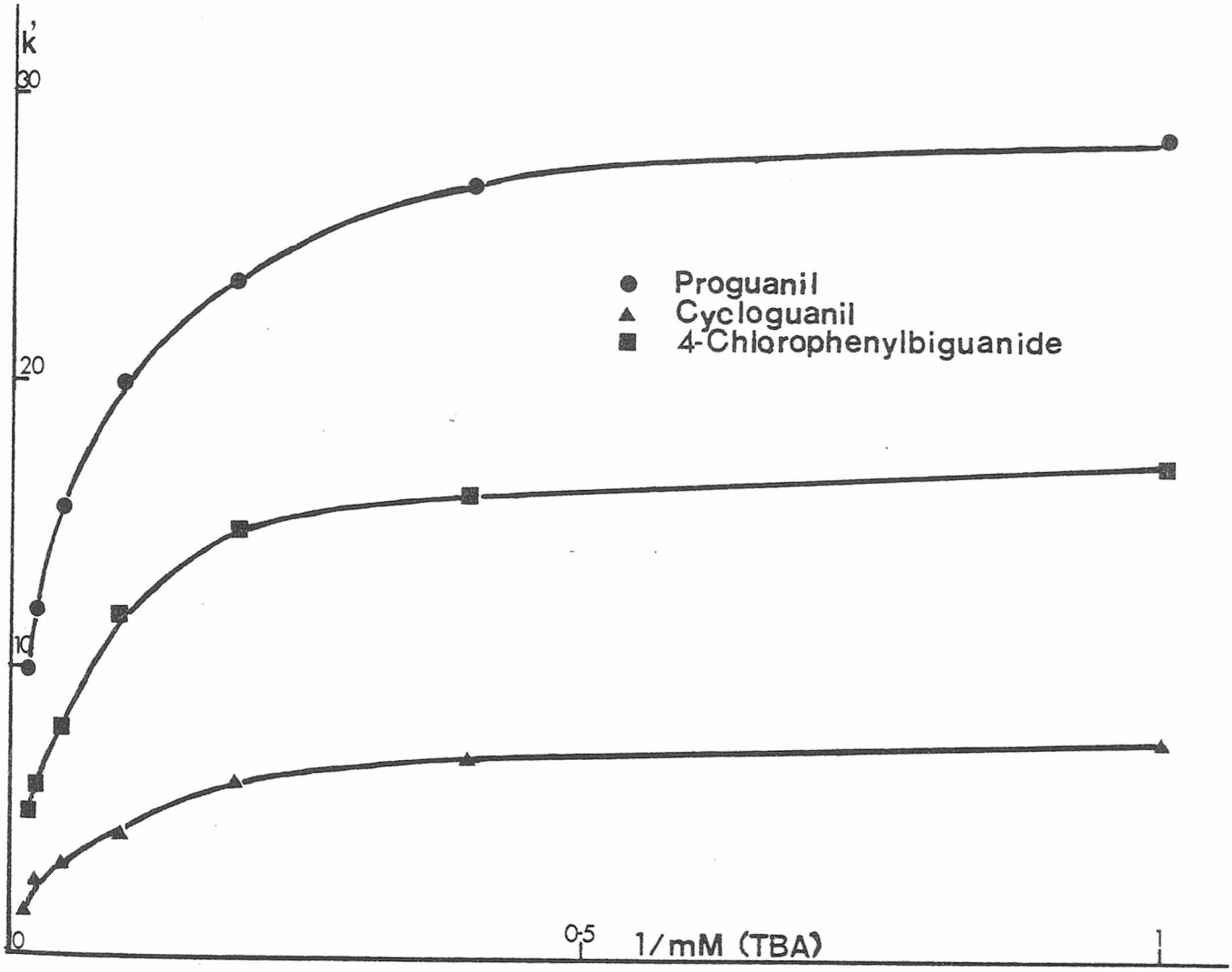


Figure 58 Plots showing the variation in capacity factor (k') of the three biguanides as a function of the reciprocal of the concentration of added (TBA) organic counter ion. Chromatographic conditions as in Figure (55).

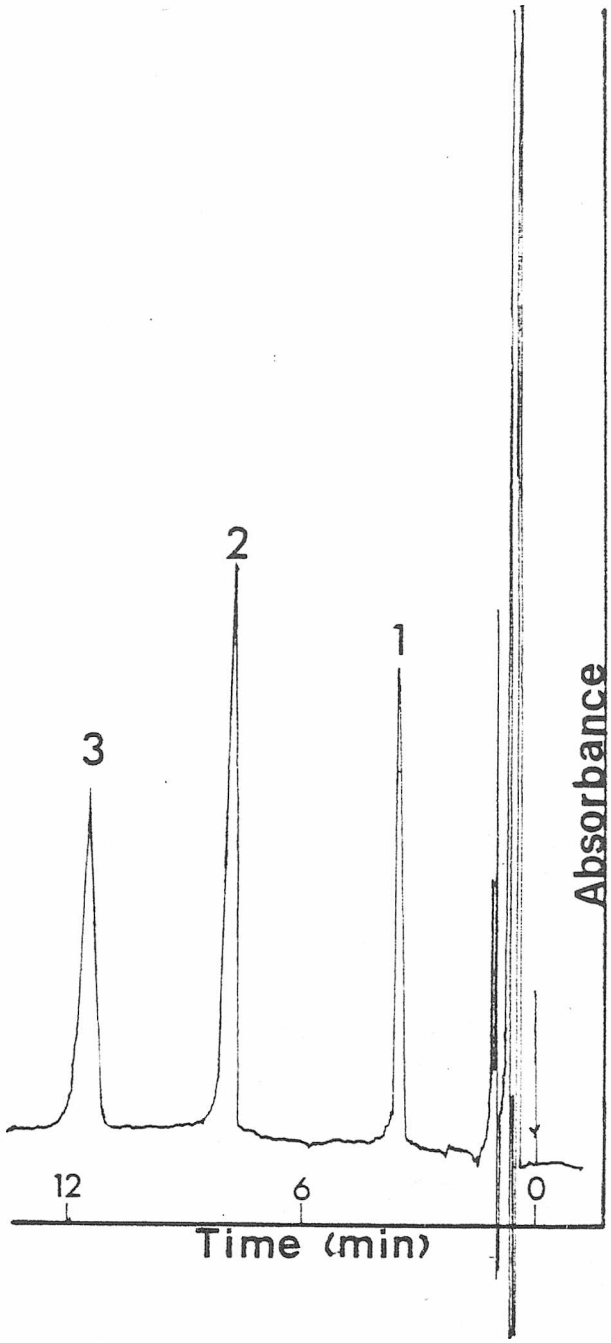


Figure 59 Chromatogram showing the complete separation of the three biguanides in presence of added organic counter ion. Chromatographic conditions: 100 x 4.6 ODS-Hypersil column, solvent 50% v/v acetonitrile-buffer of 10 mM disodium hydrogen phosphate at pH 2 with the (SLS) concentration of 90 mM with the addition of 5 mM (TBA) as organic counter ion. Flow rate at 2.0 cm^3 min^{-1}. Compounds identification as in Figure (51).

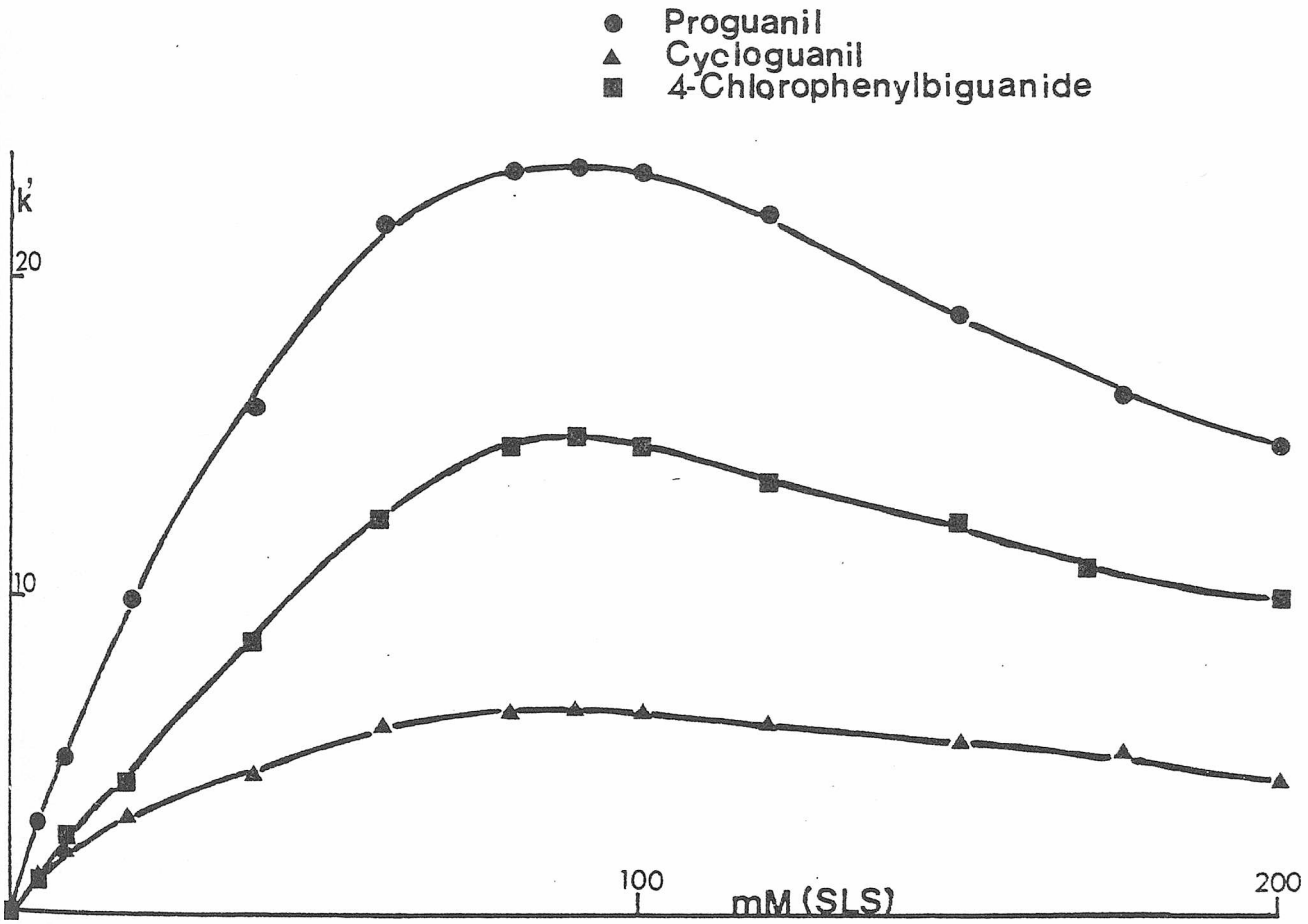


Figure 60 Plots showing variation in (k') with mobile phase pairing ion (SLS) concentration for three biguanides. Chromatographic conditions as in Figure (55) with the mobile phase modified to include 5 mM (TBA).

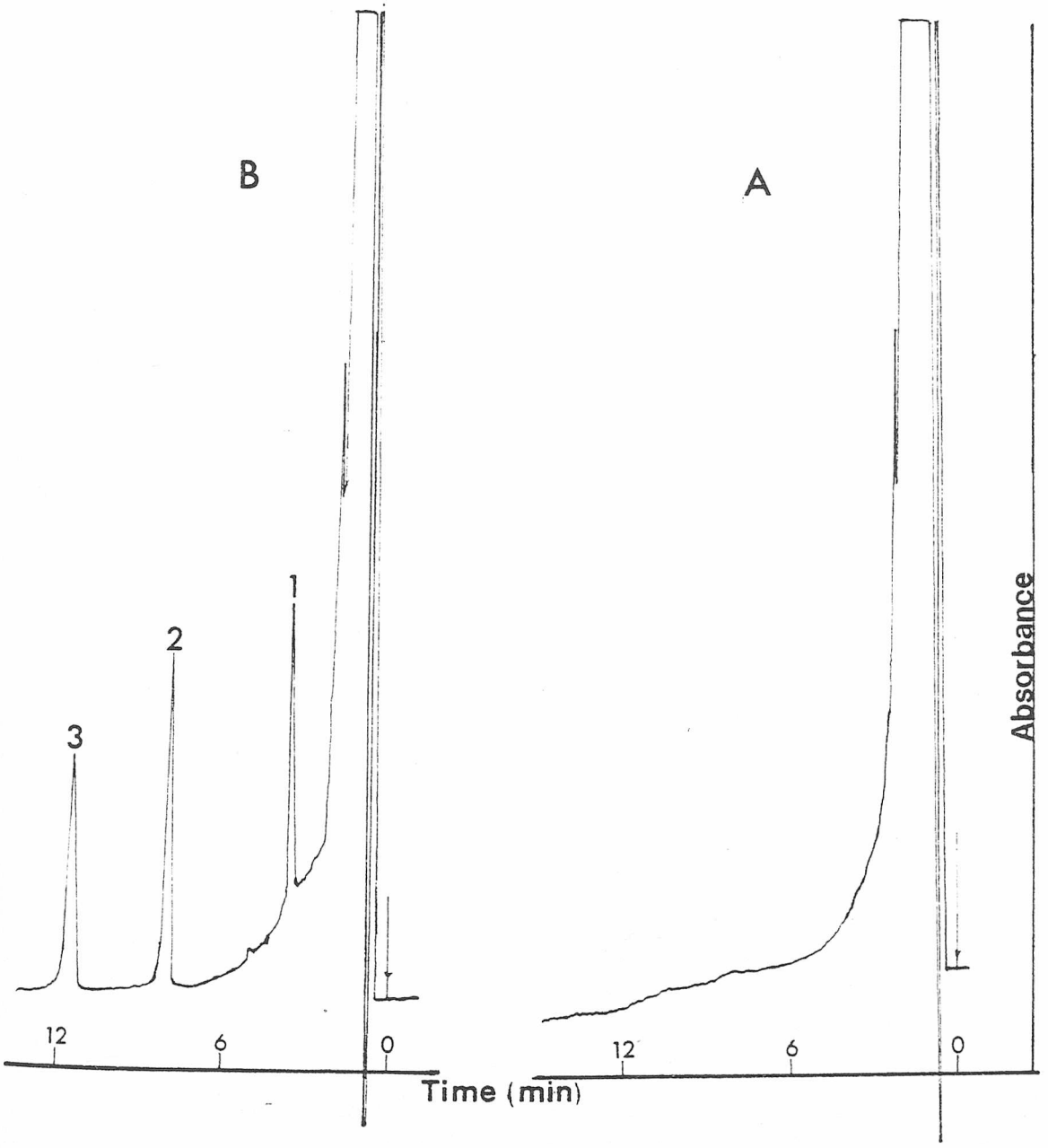


Figure 61 (a) Chromatogram of serum blank extract.
(b) Chromatogram of a serum extract containing 25 ng cm^{-3} of each of the three biguanides.

Chromatographic conditions as in Figure (59) with wavelength of detection at 254 nm at full scale deflection of 0.01 absorbance units.

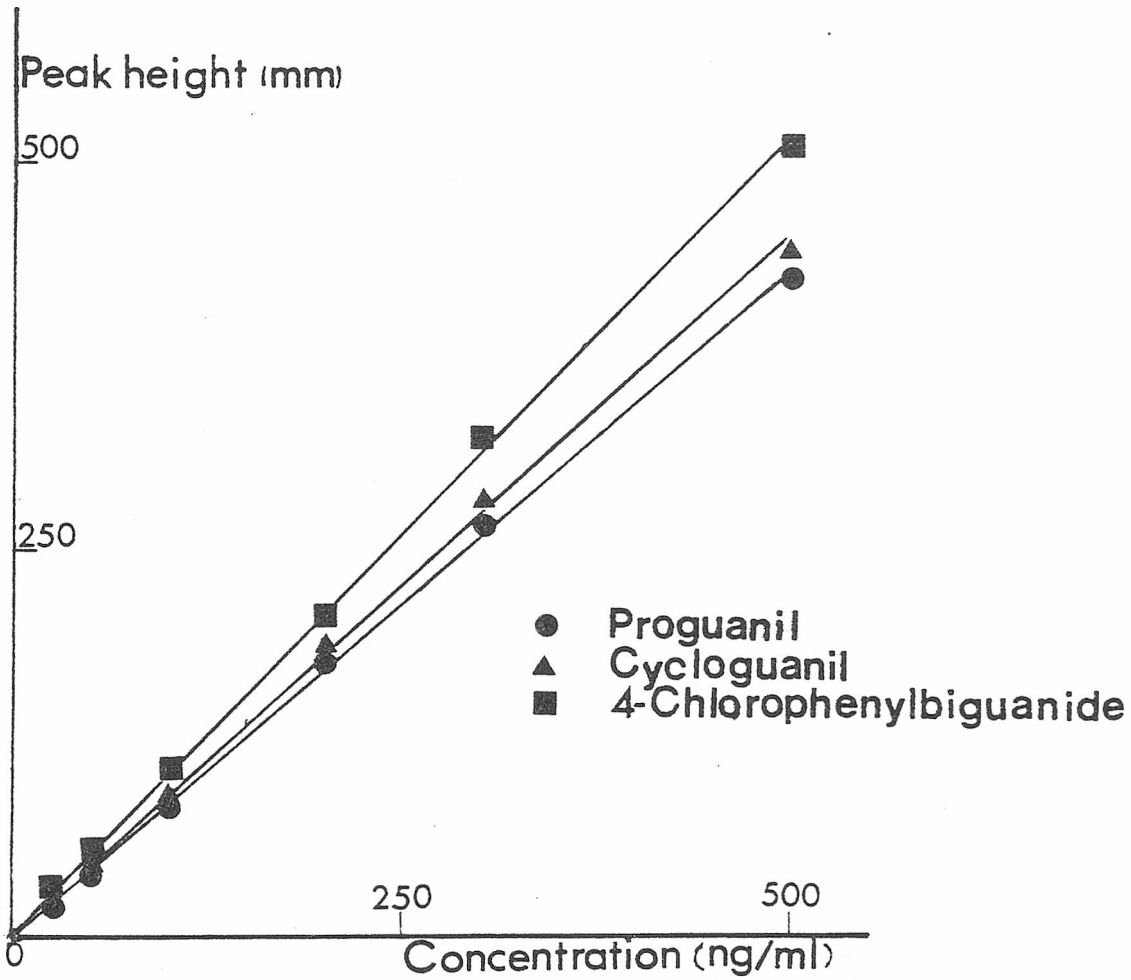


Figure 62 Calibration curves for the three biguanides, peak height in mm (corrected to full scale deflection of 0.01 absorbance units) vs. concentration for drug-free serum samples supplemented with these drugs.

Drug	Recovery % Concentration in ng ml^{-1}	
	50	250
Cycloguanil	76	80
4-chlorophenylbiguanide	84	85
Proguanil	87	87.5

Table 14

Table showing the mean percentage recovery of the biguanide bases from serum (n=4).

Drug	Slope	Y-Intercept	R
Cycloguanil	0.8975	0.3955	0.9902
4-chlorophenylbiguanide	1.0374	0.3694	0.9993
Proguanil	0.8632	0.8170	0.9994

Table 15

Table showing the linear regression data for the calibration curves of the biguanide bases.

CHAPTER SIX

A DISCUSSION OF THE PROBLEMS ASSOCIATED WITH QUANTITATIVE STRUCTURE RETENTION RELATIONSHIP (QSRR) INVOLVING BONDED REVERSED PHASE HPLC

6.1 Introduction

In Chapters (2) and (4) considerable attention was devoted to the processes which have been suggested as responsible for retention of ionised species in chemically bonded reversed phase chromatography. The interpretation of the present results was based on a combined mechanism of ion-exchange followed by desolvation of an electrically neutralised solute on the C-18 surface. Attention was focussed on the process of neutralising the electrostatic charge by previously adsorbed pairing ion on the surface of the C-18. This effect was shown to be general for many different solutes including the test solutes of simple carboxylic acids and bases, the furan acids derived from D-glucose degradation, and the tricyclic antidepressant and antimalarial drugs. Comparatively little attention was given to the desolvation element in the retention mechanism and it is this part of the retention process which was shown to allow separation to be achieved among solutes. That is, a combined ion-exchange-desolvation constant designated $K'_{des}.K_{IE}$ was evaluated which was roughly constant for all pairing ions for a given solute but different from solute to solute. This was taken as indicating a constant desolvation property for each solute largely independent of pairing ion.

The process of retention of the electrically neutral species in the present work in being described in terms of a desolvation constant is to some extent at variance with the majority of the chromatographic literature. Generally this process of retention (which will also be applicable for neutral organic solutes) is described in terms of bulk phase partitioning and the solutes described as being of varying hydrophobicity or lipophilicity [69,75,240-243].

The tacit assumption is made in many publications that the process producing retention in reversed phase chromatography is identical with that producing partitioning of a solute between pairs of immiscible liquids.

Differences in retention among solutes, producing separation, have been assumed to be caused by differences in the particular equilibrium constant for such a process usually termed the partition coefficient [244-248].

The converse of the above is even more apparent in the literature namely that if the difference in retention among a set of compounds is measured by a reversed phase chromatographic technique, then such differences are a measure of the differences in partition coefficients [69,75,240-248]. This reasoning is taken further and it is claimed that measurements of chromatographic reversed phase retention parameters e.g. k' or Rm values provide a source of data on the partition coefficients of the measured solutes [246-249].

If such an approach, which will be expanded more fully below, is valid i.e. if partition coefficients for different compounds can be determined experimentally from chromatographic parameters using reversed phase systems it follows that, from a knowledge of the partitioning behaviour of a set of compounds their order of elution is predictable in reversed phase chromatography.

It is the purpose of the present chapter to review and comment on both the literature data and data obtained in the present work concerning the interrelationship between physical properties of solutes, in particular their hydrophobicity, and their chromatographic behaviour. The existence of such interrelationships and a knowledge of their form would have a twofold advantage.

1. The process determining retention and selectivity would be better understood chromatographically.
2. Chromatographic separations could be predicted from a knowledge of the appropriate physical constants of the solutes which in many cases are calculable.

Historical

In the study of quantitative structure activity relationships of drug molecules (QSAR) it has always been realised that the hydrophobicity of the drug was important [250-256]. Firstly because such a property was important in the transport of a drug in a biological system to the active site and also because certain drug receptor interactions were believed to be hydrophobic in nature [257,258]. In the early days of QSAR the assumption was made that bulk phase partition coefficients provided the best or perhaps the only estimate of hydrophobicity.

Because of the time consuming nature of direct experimental coefficient measurement, alternative methods were applied to obtain such information very prominent among which was reversed phase chromatography [259]. The procedure adopted from such reasoning took two main directions.

1. Attempts were made to correlate chromatographically determined quantities such as Rm with measured biological activity. Such attempts clearly implied that the interactions being studied in both experimental systems were related if not identical [248,249,259].
2. Attempts were also made to correlate chromatographic variables with measured partition coefficients perhaps as justification for their use in QSAR [244,246].

Much of the earlier work in this field predated the advent of HPLC and was carried out on reversed phase, thin layer systems [259-261]. The validity of the approach was taken as being justified by the apparently adequate correlations obtained between the measured variables. The very large assumption that partition or any property associated with partition was largely independent of the solvent system used has been explored extensively in the compilation of partition data by Hansch et al. [262] but rests to a large extent on very early work of Boyce and Milborrow [259] and

Collander [263,264]. That is, it is regularly the case that partition coefficients or biological effects measured with respect to one pair of solvents are correlated with chromatographic results obtained in a completely different set [248,249].

With the advent of HPLC this technique seemed ideal to continue this work because of the availability of high precision retention data and correlations between k' or ln k' and partition coefficients or biological activities began to appear in the literature [246,247]. The application also seemed attractive because chemically bonded phase systems especially C-18 coupled with essentially aqueous solvents appeared to offer partitioning systems very analogous with those believed to be operating in biological systems [265].

This approach has not been without criticism. Tute [266] has pointed out the pitfalls in the indiscriminate use of such correlation equations without adequate consideration of the molecular structures involved and Mirrless et al. [267] have actively criticised the basic assumption that there is any relevance in the comparison of C-18 reversed phase retention data and partition coefficient data in octanol. In chromatographic fields of study the nature of the forces producing retention and separation have remained ill-defined and vague on a quantative level. The very nomenclature of terms as basic as adsorption and partition have been discussed with editorial comment in the Journal of Chromatography [268]. It is perhaps because of this lack of understanding of chromatographic interactions coupled with the wealth of information relating physical properties to chromatographic behaviour that publications have began to appear in the literature attempting direct correlation between retention behaviour usually on chemically bonded reversed phase systems and equilibrium properties of solutes which are directly measurable or calculable [269,272].

In the chromatographic literature two main approaches are apparent.

One is exactly analogous to QSAR and has indeed led to the coining of the analogous term of quantitative structure retention relationship (QSRR) [273,274]. The other involves a priori attempts, on a theoretical basis, to quantify the capacity factor of a solute in terms of the various interactions believed to be present in a chromatographic system [71]. The latter approach as will be indicated below involves many physical properties and involved relationships and, perhaps as a result has received little general testing on an experimental basis.

It is appropriate at this time to evaluate these approaches with a view to establishing how a better understanding of chromatographic retention process can be achieved. Such an evaluation will be limited to the chromatographic measurements involving high pressure liquid chromatography utilising chemically bonded reverse phases.

6.2 Results: Attempts to relate capacity factor with single properties depending on hydrophobicity

It is generally assumed that the hydrophobicity of a solute determines, to a large extent, its retention in BRPHPLC systems. In attempts to verify this assumption, several criteria of such hydrophobicity have been suggested. These include the Hansch π [141] as a substituent constant indicating group contribution. The logarithm of the partition coefficient (log P in octanol/water) is also taken as a measure of the hydrophobicity of the total molecule. Both of these parameters have been extensively used in QSAR studies [275-277]. More recently a topological index χ, has been derived by Randic [278] and has been shown to relate to several physical and hydrophobic properties of a molecule. All of the above properties have been shown to be highly correlated one with another [279-281].

Recently several articles have been published dealing with such relationships [101,115,282-286]. Good correlations have been demonstrated

between measured log k' values of the solutes examined chromatographically and their derived hydrophobic constants. Some of these results will be discussed below.

It has been demonstrated by Hearn and Hancock [284] that the log P of a series of substituted thyronine compounds has a linear correlation to their log k' values as shown in Figure (62). Such a plot has also been reported by Braumann and Grinime [285] on a series of substituted pyridazinone compounds as shown in Figure (64). The regression lines of their plots are listed below.

$$\log k' = 0.3146\ (\pm\ 0.0433).\log P - 0.7764\ (\pm\ 0.16757) \qquad -(59)$$

$$R = 0.9395 \text{ (Ref. 284)}$$

$$\log k' = 0.6041\ (\pm\ 0.0684).\log P - 0.8711\ (\pm\ 0.13474) \qquad -(60)$$

$$R = 0.9637 \text{ (Ref. 285)}$$

Linear correlation between the log k' of a series of alkyl alcohols with their χ has been reported by Colin and Guichon [115] as shown in Figure (65) and the regression line is;

$$\log k' = 0.6565\ (\pm\ 0.0700).\ \chi - 1.7181\ (\pm\ 0.19087) \qquad -(61)$$

$$R = 0.9727 \text{ (Ref. 115)}$$

The correlation between the log k' of a solute and some derived hydrophobic index has not only been limited to neutral compounds. Recently Tomlinson et al. [101] has used a term $\mathcal{T}$ which is given by log $\log(k'_j/k'_i)$, where k'_j and k'_i are the capacity factors of a substituted compound j and the nonsubstituted compound i. It was demonstrated that $\mathcal{T}$ is directly proportional to the difference in hydrophobicity, in terms of π, between substituted and nonsubstituted compounds.

For a series of substituted benzoic acids the relationship [101],

$$\log (k'_j/k'_i) = \mathcal{T} = 0.49\pi + 0.03 \qquad R = 0.94 \qquad -(62)$$

was obtained under ion pairing conditions.

Equation (62) can be rearranged to give,

$$\log k'_j = m\pi + (C + \log k'_i)$$

where m and C are constants; as is $\log k'_i$.

When the results of the same set of substituted benzoic acids are plotted as a function of π a much inferior correlation is obtained. This is shown in Figure (66) and results in a correlation of the form,

$$\log k'_j = 0.3125\ (\pm\ 0.0705).\ \pi\ + 0.4437\ (\pm\ 0.0352) \qquad -(63)$$
$$R = 0.7759$$

It would appear from these facts that, the normalisation of the capacity factors by use of the retention associated with the unsubstituted compound markedly reduces the variance of the set of data.

The few examples cited above would indicate that while correlations exist between log k' and log P or χ for neutral compounds in a closely related group, such agreement does not occur in the case of equally closely related compounds under ion pairing conditions using π as the hydrophobic parameter. The above examples however of neutral compound sets include only closely related solutes. Previous unpublished work in this laboratory [287] in a set of substituted benzene derivatives on Partisil ODS yields the results shown in Figure (67A). A linear relationship of the form

$$\log k' = 0.3854\ (\pm\ 0.06265).\log P - 0.2371\ (\pm\ 0.10884) \qquad -(64)$$
$$R = 0.8627 \text{ was obtained}$$

It can be observed from Figure (67A) that a wide range of relatively different compounds was examined. This correlation can be markedly improved by the judicious omission of certain compounds, and Figure (67B) shows the results obtained by limiting the number of compounds studied.

A linear relationship of the form

$$\log k' = 0.3226\ (\pm\ 0.02597).\log P - 0.1117\ (\pm\ 0.04744) \qquad -(65)$$

$R = 0.9721$ was obtained.

Verification of the above fact that correlations obtained in the literature depend very much upon the choice of compound can be obtained from the work of Tanaka et al.[288]. This study was concerned primarily, not with the problem of correlating retention and physical characteristics for solutes sets, but with investigating differences in selectivity among several bonded stationary phases incorporating different alkyl chain lengths ranging from C1 to C18. A very wide range of solutes were employed. From this publication, it was possible to abstract data on log k' and to calculate χ_i for each solute[288]. The data chosen as representative was that obtained using Develosil C-8 as stationary phase with methanol-water (80:20) mobile phase. The results obtained are shown in Figure (68). Even allowing for some lack of precision in the log k' valves taken from the plot of log k' for C-8 versus log k' for C-1 in reference (282), Figure (68) nonetheless shows an almost complete lack of correlation

$$\log k' = 0.06252\ (\pm\ 0.02532).\chi + 0.19594\ (\pm\ 0.13050) \qquad -(66)$$

$R = 0.4929$

Good correlations, however, can be obtained if the analysis confined to selected compounds e.g. compounds 5, 6, 7, 8, 13 and 14 provide a very good linear relationship. In this group the first four constitute a homologous series and all of them are saturated hydrocarbons. If aromatic and polynuclear hydrocarbons are considered e.g. compounds 9, 10, 11, 12, 16, 17, an equally good linear plot is obtained. Also compounds 1, 2, 3 and 4, all alkyl substituted benzenes show very adequate linearity.

These results emphasise that while correlations between log k' and the

accepted hydrophobicity parameters can be obtained, they are limited to compounds which are very similar in structure and that the correlation is not adequate in any general sense.

In Chapter (2) retention of ionic compounds was suggested as occurring by a combined process of ion-exchange coupled with desolvation. It was further indicated that selectivity among solutes was as a result of the desolvation part of this mechanism. Relatively few results have appeared in the literature relating retention to hydrophobicity for ionised solutes and the results on the substituted benzoic acids [101] have already been reviewed. The results obtained for the tricyclic anti-depressant drugs in Chapter (4) provide a source of data on a set of bases where the basic chemical structures are very similar but which have marked differences in substitution pattern and type. The log k' values for these compounds at the maximum in their retention pattern i.e. at 80 mM SLS in 50:50 acetonitrile-buffer at pH 2 is plotted against the calculated χ_i value in Figure (69).

The χ value is used since it appears to be gaining general acceptance as a hydrophobicity parameter [115,281,282] and is easily calculated from a knowledge of the molecular structure only. The corresponding total molecular property of log P could not be readily obtained from the literature for these compounds and application of the additivity principle of substituent constant π values was avoided in view of the inherent unreliability of this process [266]. The regression line corresponding to the plot in Figure (69) is,

$$\log k' = 0.07934\ (\pm\ 0.08195).\chi + 0.7373\ (\pm\ 0.6582) \qquad -(67)$$

$$R = 0.2928$$

indicating a complete lack of any statistical correlation. Such a finding is not totally unexpected since the overall similarity in size and shape

of the compounds in this group gives rise to a very small range in values (7.4 to 9) while there are very considerable differences in retention among the members of the series as shown in Figure (36). The lack of correlation is further emphasised by noting that amitriptyline has a much larger χ than nortriptyline but has in fact shorter retention time. Similar effects are observable for the solute pair imipramine and desipramine.

It would appear from the above evidence that either hydrophobicity is not the determinating influence in determining retention of the solutes in chemically bonded reversed phase systems or that the parameters used to quantify the hydrophobic nature of a solute do not accurately measure this property. It is however the case that such correlation are regularly published in the literature. The best that can be claimed is that such correlations are obtained when a very similar set of closely related neutral compounds are examined. It would appear from the above examples that it would be very suspect to attempt prediction of chromatographic behaviour based on estimation of any simple parameter currently used to estimate hydrophobicity.

6.3 Discussion

The fundamental question underlying any discussion concerning such relationships between chromatographic retention and molecular property is that of mechanism of the retention process. If again the discussion is limited to the extensively used BRPHPLC, there have been three main retention mechanisms suggested.

It has been argued that retention of solutes in such chromatography involved partitioning of the hydrophobic solute into the quasi-solution bonded phase [69]. Knox and Pryde [289] modifying this idea, suggested that the bonded silica gel simply provides a surface for the adsorption of a

liquid stationary phase which consists of the organic modifier and water and that the composition of this adsorbed liquid phase is different from that of the mobile phase. Partitioning of the solute molecules is suggested as taking place between the mobile phase and adsorbed liquid stationary phase. Another possible retention mechanism is adsorption [290-293] of the hydrophobic solute on the non-polar stationary surface. The interaction between the solute and the stationary phase would be expected to be rather weak, depending as it does only on the non-specific dispersion process of the Van der Waals type. The third possible retention mechanism is the solvophobic adsorption mechanism. Such a process has been extensively studied by Horvath et al. [71] and has received support from numerous workers [70,115,282]. In this mechanism the retention of the hydrophobic solute is envisaged as being a mobile phase effect where a solute is squeezed out from the polar mobile phase onto the non-polar stationary phase due to the so-called solvophobic force.

If partition is the retention mechanism in BRPHPLC, then the more lipophilic the molecule the longer will be its retention. Thus addition of a lipophilic group onto a molecule will increase its retention due to the group contribution effect as suggested by Martin [294]. Martin suggested that a substituent changes the partition coefficient of a substance by a given amount that depends on the nature of the substituent and the two phases employed, but not on the rest of the molecule.

However, if adsorption or solvophobic process determine retention in BRPHPLC, retention of a moleclue may not only depend on its lipophilicity. In adsorption and solvophobic adsorption process, the attraction between the solute and the stationary phase depends only on the contact surface area between them. The addition of a lipophilic group may not cause any increase in the contact surface area between the solute and the stationary phase. The ideas of partition and adsorption are oversimplified

in that they consider only interactions between solute and stationary phase and it is becoming increasingly realised that retention depends upon solute-solvent and solvent-adsorbent interactions as well as those between solute and adsorbent.

In order to predict the retention of a solute in any chromatographic system in the reversed phase mode, an equation relating the k' of a solute must be formulated as a function of solute, solvent and adsorbent properties i.e;

$$k' = f(\text{solute}) + f(\text{solvent}) + f(\text{adsorbent}) \qquad -(68)$$

Only the solvophobic adsorption model has been studied extensively and an equation with a form similar to that of equation (68) been derived. According to the solvophobic mechanism [71], the k' of a nonionised solute may be obtained from the following expression;

$$\ln k' = A + B.\mathcal{D} + C.\gamma + D.(\mathcal{K}^e - 1).V^{\frac{2}{3}}.\gamma + E + \ln(RT/PoV) \qquad -(69)$$

The various variables $\mathcal{D}$, γ, $\mathcal{K}^e$, V and E refer to various solute and solvent properties and will not be considered in detail here.

The complexity of equation (69) indicates that it is rather naive to use a single derived constant e.g. π, χ or log P to represent the right hand side of the equation. If the solvophobic adsorption mechanism adequately explains retention in BRPHPLC, then the k' of a non-ionised molecules can be calculated by equation (69) provided that all the variables on the right hand side of the equation are known. Unfortunately, quantitative treatment of the retention of a solute as a function of solute, solvent and adsorbent properties, in the light of the solvophobic mechanism as expressed in equation (69) has not been considered seriously by many chromatographers.

Instead, most attention has been focussed on the simplified version

of equation (69) shown below. According to Horvath et al. [71], for a given solute the effect of changing eluent composition can be expressed by equation (69), when only the surface tension changes.

Equation (69) can be simplified to,

$$\ln k' = A''' + \frac{\gamma.N.\Delta A + \gamma.4.836.N^{\frac{1}{3}}.(\kappa^e - 1).V^{\frac{2}{3}}}{R.T} \qquad -(70)$$

where A''' is the sum of all terms which do not contain the surface tension. γ, N, ΔA are the surface tension of the solvent, Avogardro's Number and the contact surface area between the solute and the adsorbent respectively. κ^e expresses the ratio between the energy required for the formation of a suitable shape cavity of the solute in the solvent and the energy required to expand the planar surface of the solvent by the same area. V is the molar volume of the solvent.

Equation (70) is certainly incorrect in making the assumption that changing eluent composition will only affect the surface tension of the mobile phase. It has been demonstrated by numerous workers [295-297] that changing the eluent composition by altering the concentration of the organic modifier or by using different type of organic modifier or by addition of another organic solvent as in the case of ternary mobile phase systems will cause marked changes in selectivity of the chromatographic system. These can reasonably be explained on the basis of alterations in solute-solvent interactions and solvent-adsorbent interactions.

It is more likely, however, that equation (70) is correct in predicting that ln k' of a solute is directly proportional to the contact surface area between the solute and the adsorbent rather than the total surface area of the solute. A spherical molecule may have an identical surface area to a planar molecule, but its contact surface area with the bonded phase will certainly be different. Also the term κ^e can not be regarded as a constant when the shapes of two solute molecules are

appreciably different. Thus,the shape of a molecule is also an important factor in determining the retention of the solute. It is notsurprising, therefore, to find out that nearly all the data published demonstrating a linear relationship between the ln k' with the derived physical constants e.g. π or χ of the solutes are confined to a homologous series or a congeneric series of compounds with similar shape. Where a homologous series of compounds is used, their shapes will be very similar and thus their contact surfact areas with the adsorbent can be adequately represented by their total surface areas as represented by χ or π [298] and also κ^e among the series can be regarded as a constant.

If compounds of widely differing molecular structures are considered the ln k' of the solutes may not show a linear dependence on the χor π values as indicated in Figure (68). Recently Wise et al. [299] have introduced the use of the length and breadth ratio to indicate the shape of a molecule, together with the χ to represent the hydrophobicity of the molecule in order to overcome the above problem in quantitative structure retention studies. Such a length to breadth ratio represents a very crude attempt to quantify shape but indicates that shape is considered to be of some importance in the retention process.

It is by no means certain that equation (69) accurately represents all of the factors determining the k' value of a solute in a chemically bonded reversed phase system. The description is incomplete in that it does not explain the observation that carbon chain length of the bonded alkyl ligand affects the selectivity as well as the capacity factor in reversed systems [69,70]. Furthermore because of the use of different bonding techniques during the synthesis of reversed phase silica, there are two distinctly different types of bonded phases; namely the bulk type and the bristle type. In the bulk type stationary phase, di- or tri-functional organo-silanes are used to react with the silica gel which allows

polymerization of the ligands to occur. The bulk type stationary phase can thus be regarded to behave as a quasi-solvent as suggested by Lochmullar et al. [69,300]. In the case of the bristle type stationary phase, monofunctional organosilanes are used to react with the silica gel so that no polymerization takes place. As suggested by Berendsen et al. [70], such stationary phases may possess certain steric properties where a long and thin molecule can desolvate on the C-18 surface by inserting itself into the gaps created by the bristle type C-18 "jungle". A large bulky molecule, on the other hand, will be less efficient in desolvating itself on the hydrophobic stationary phase because of its size and shape; that is, it cannot squeeze itself into the gaps suggested as existing between adjacent C-18 chains. On the other hand the C-18 ligand is not a rigid structure; it can rotate, producing different conformations. Thus it can bend itself when it comes into contact with a spherical molecule resulting in an increase in available contact surface area.

It has also been reported that the bristle-like reversed phase can have different conformations in different mobile phase system [143]. In an eluent of high water content, the C-18 ligands will collapse and aggregate with each other to form C-18 droplets, which may resemble the structure of bulk-type bonded phases. In mobile phase systems with a high content of organic modifier, the C-18 ligands resume their bristle-like structure. In other words, if this type of stationary phase is used, the adsorbent property is strongly dependent on the eluent employed. In practice, the two types of reversed bonded phase can further be subdivided into capped an non-capped bonded silica gel. While such non-uniformity of bonded reversed phase will certainly increase the versatility of application of reversed phase chromatography it also creates problems in quantitative retention studies in this field. Although reversed bonded phases are used so successfully, they also constitute one of the least well defined

systems in liquid chromatography. As stated by Karch [293], a well defined and reproducible stationary phase is required before one can begin to understand mobile phase or bonded phase effects.

Horvath et al. [73] has also suggested that the ideas of solvophobic retention can account for the retention of ionic hydrophobic solutes on bonded reversed stationary phases. In the case of ion-pair chromatography, however, the situation becomes very complex, as addition of pairing ion will concomittantly alter the nature of the stationary and mobile phases. The electrostatic property of the solutes under ion pairing conditions is as important as their hydrophobic properties. For example, a negatively charged solute will be repelled from a stationary phase which has been modified by adsorbed anionic hydrophobic pairing ion. Even the retention of a neutral solute can be affected in ion-pair chromatography as demonstrated in Chapter (2) using 5HMF as the test solute. Another problem involved in QSRR in ion-pair chromatography is that the elution order of the compounds can be varied with the mobile phase pairing ion concentration, as demonstrated in the retention of several different ionic solutes in Chapter (2), (3), (4) and (5) using different pairing ions. This has the significance that, even though a correlation between the log k' and the χ or π of the solutes at a particular pairing ion concentration could be obtained, the same correlation might not be apparent at any other pairing ion concentration. Also different selectivities among solutes on altering the type of pairing ion used have been reported [153].

6.4 Conclusions

The above observations highlight the complexity of the reversed phase retention process particularly in the context of ionic solutes when pairing ion is also involved. They cast considerable doubt on the likelihood of ever achieving meaningful correlations between the capacity factor and

any single property purporting to be a measure of hydrophobicity. Such correlations as have been obtained, have been shown to be very limited in the extent of differences in molecular structure explored. It is difficult to find in the literature appreciable evidence that such single parameter correlations have any real degree of generality.

To a large extent the reason of the lack of meaningful correlation between capacity factors and physical properties lies in the difficulty of describing the mechanism or processes responsible for retention. Coupled with this is the fact that very crude physical parameters are generally employed possibly on the basis that they have achieved acceptance in the field of drug quantitative structure activity relationships. A fundamental fallacy would appear to be that a bulk phase property, the partition coefficient, representing an equilibrium constant between two phases is pressed into service to describe interactions which are increasingly believed to occur between surfaces. While it may be that both the bulk phase process and the surface desolvation process are controlled by the same basic solute solvent interactions, the role of the absorbent in terms of its physical and chemical behaviour cannot be ignored.

It would appear that while estimates of hydrophobicity have been obtained by measurement of chromatographic variables these have depended on the assumption that the process of retention is one of bulk partition. This may be the case in reversed phase thin layer chromatography, although there is no reason to exclude similar solvophobic effects to these described above. When the concept is extended to include modern high resolution bonded reversed phase materials the assumption becomes much less plausible. The very selectivity obtained using such stationary phases leads to the conclusion that factors other than hydrophobicity play important parts in solute retention. That is the size and shape of

the molecule, neither of which is adequately represented by a hydrophobic parameter, will have particular effects on retention due to the unique physical characteristics of different C-18 surfaces.

It thus seems unlikely that any of the approaches considered above can readily be extended to provide a predictive capacity as to how retention is linked with structure however desirable this goal may appear.

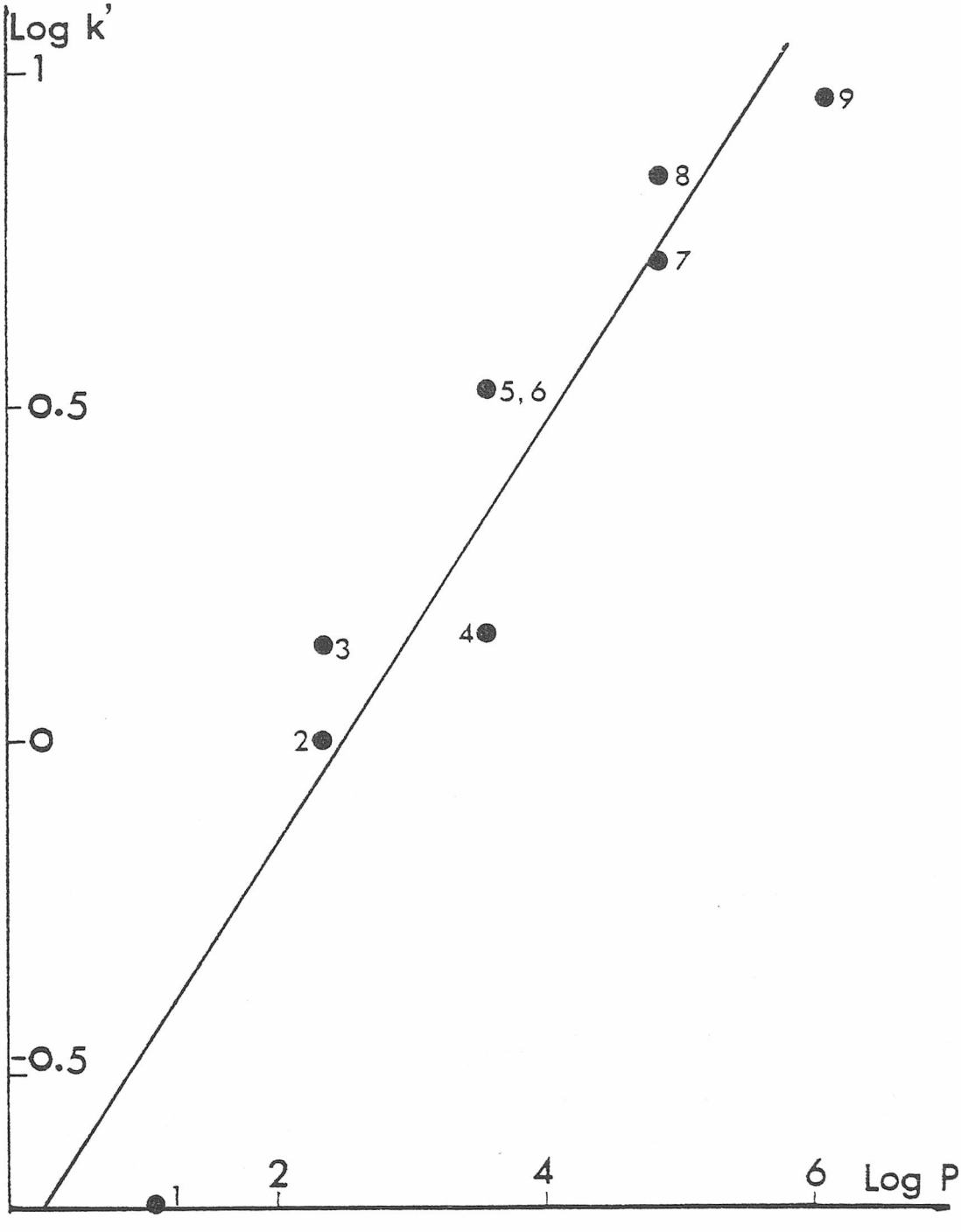


Figure 63 Graph showing the thyronine log k' values vs. log P values. Chromatographic conditions: stationary phase μ Bondapak C-18, mobile phase 50% v/v methanol-buffer mixtures at pH 3. Compound identification: 1-thyronine, 2-3-monoiodothyronine, 3-3'-monoiodothyronine, 4-3,5-diiodothyronine, 5-3',5-diiodothyronine, 6-3,3'-diiodothyronine, 7-3,3',5-triiodothyronine, 8-3,3',5'-triiodothyronine, 9-3,3',5,5' tetraiodothyronine, (Ref. 284).

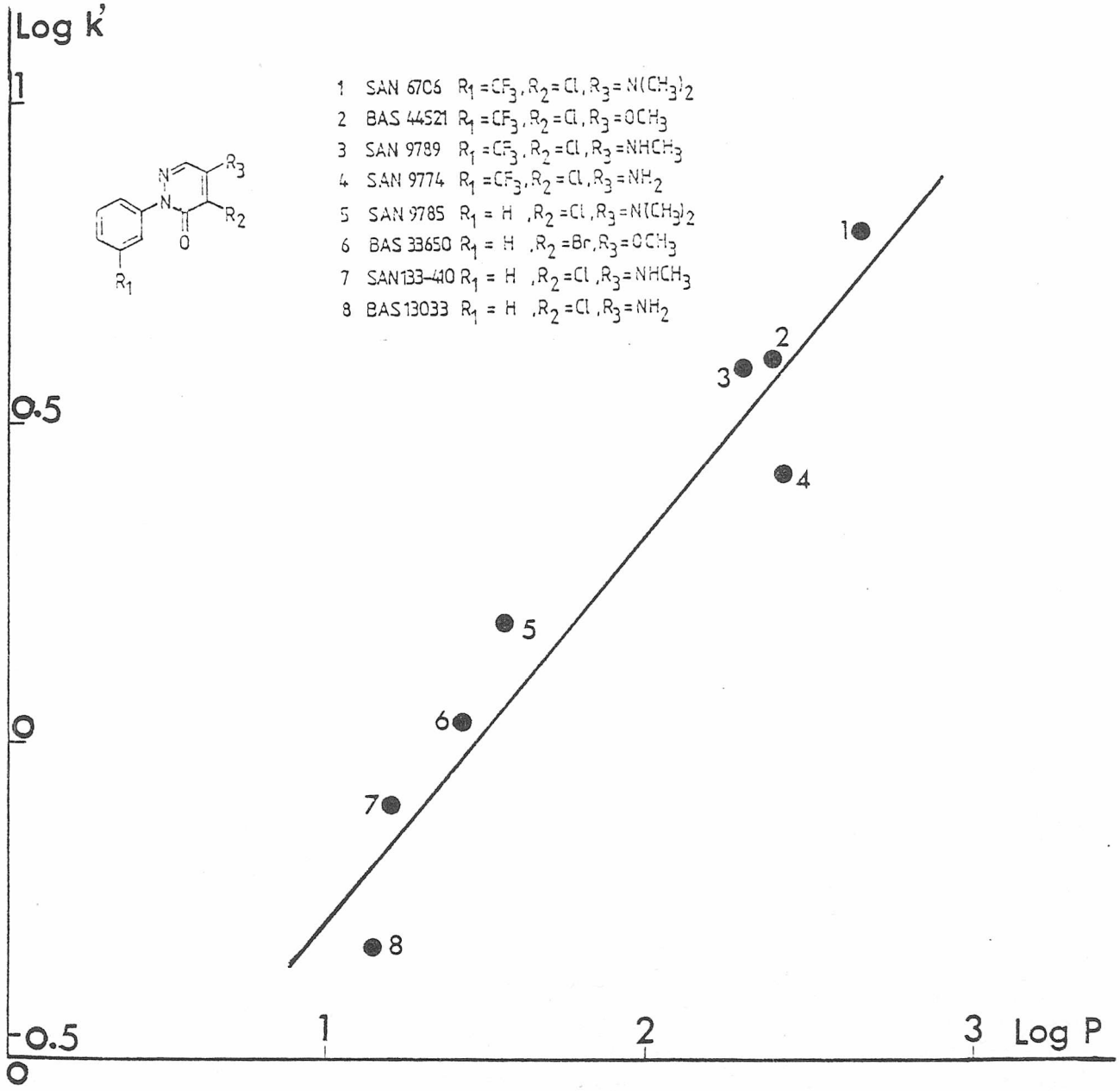


Figure 64 Graph showing log k' vs. log P for the eight pyridaziones. Chromatographic conditions: stationary phase Lichrosob RP-18 10 μm, mobile phase 55% v/v methanol-water mixtures, (Ref. 285).

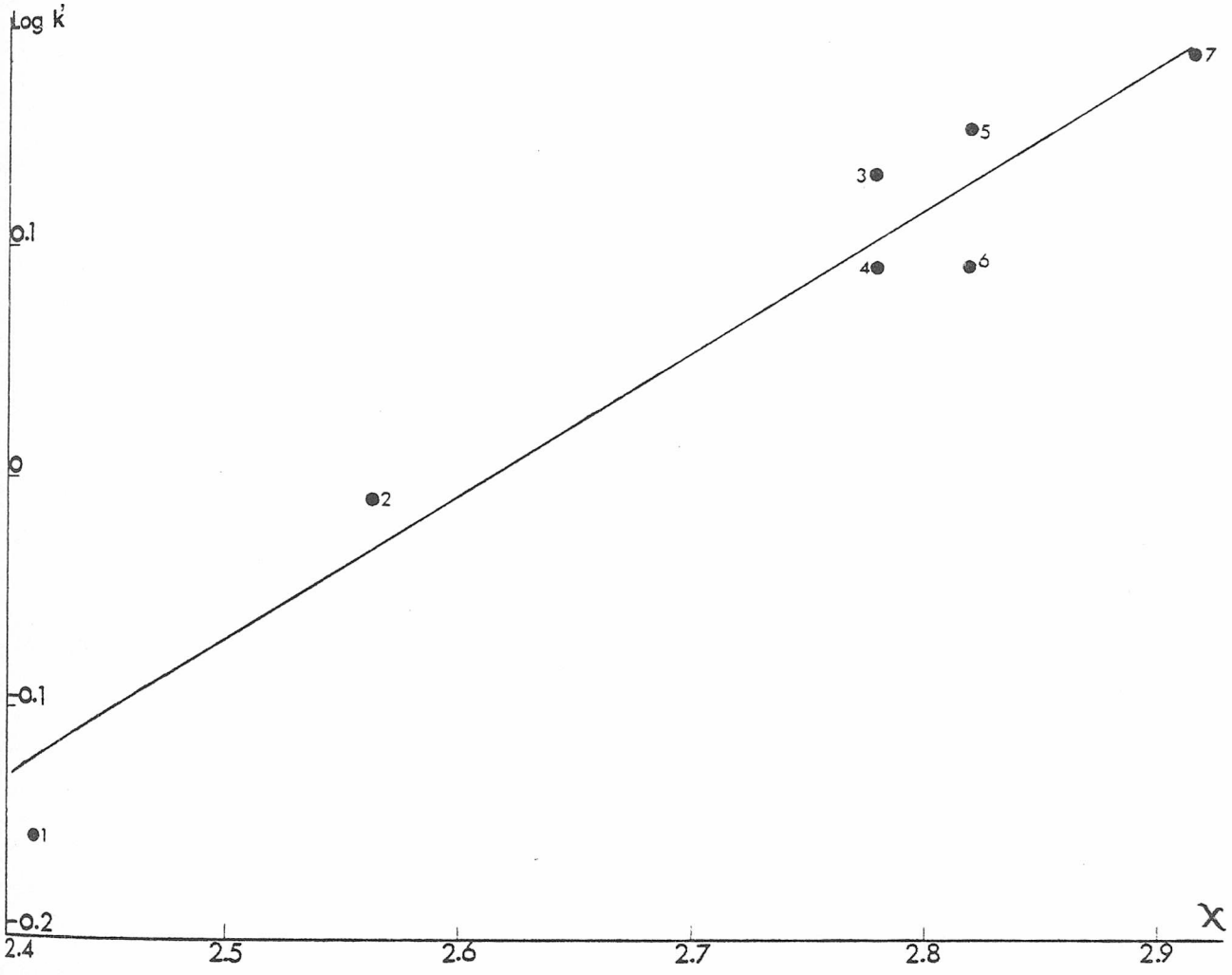


Figure 65 Correlation between log k' and χ for several aliphatic alcohols. Chromatographic conditions: stationary phase Lichrosorb RP-18 10 µm. mobile phase 58% v/v methanol-water mixtures. Compound identification: 1- n-butanol, 2- 2-methyl-2-butanol, 3- 3-methyl-1-butanol, 4- 2-pentanol, 5- 2-methyl-1-butanol, 6- 3-pentanol, 7- n-pentanol, (Ref. 115).

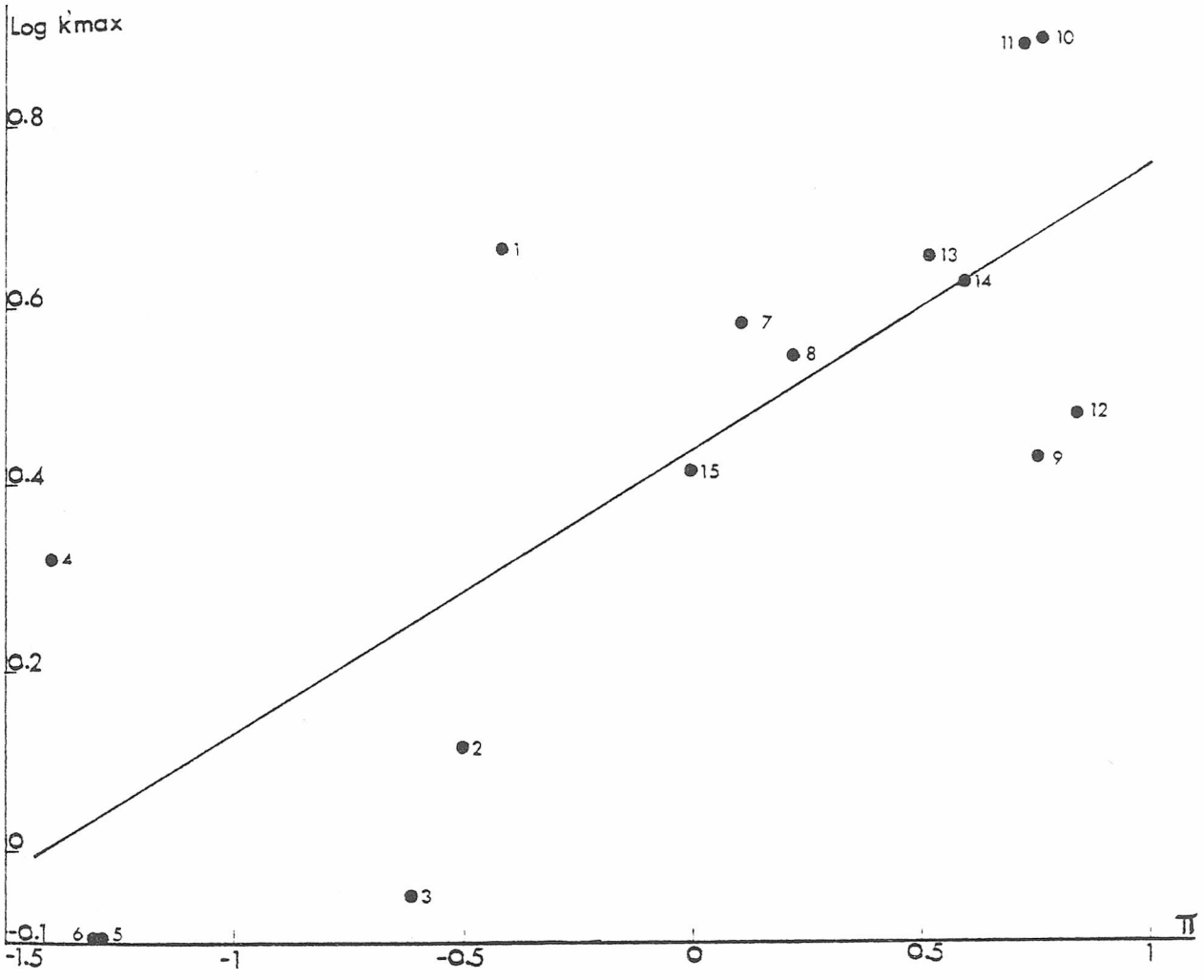


Figure 66 Correlations between log k' and π for several substituted benzoic acids under ion-pairing conditions. Chromatographic conditions: stationary phase Spherisorb ODS, mobile phase methanol-water (1:1), tetradecylbenzyldimethylammonium chloride ($0.93-7.48 \times 10^{-4}$ M), KH_2PO_4 (4.76×10^{-3} M), Na_2HPO_4 (2.02×10^{-3} M); pH 7.5; 30°C; flow-rate 2.0 $cm^3.min^{-1}$. Substituent group identification: 1- 2-OH, 2- 3-OH, 3- 4-OH, 4- 2-NH_2, 5- 3-NH_2, 6- 4-NH_2, 7- 3-NO_2, 8- 4-NO_2, 9- 2-CL, 10- 3-Cl, 11- 4-Cl, 12- 2-CH_3, 14- 4-CH_3, 15- H (benzoic acid), (Ref. 101).

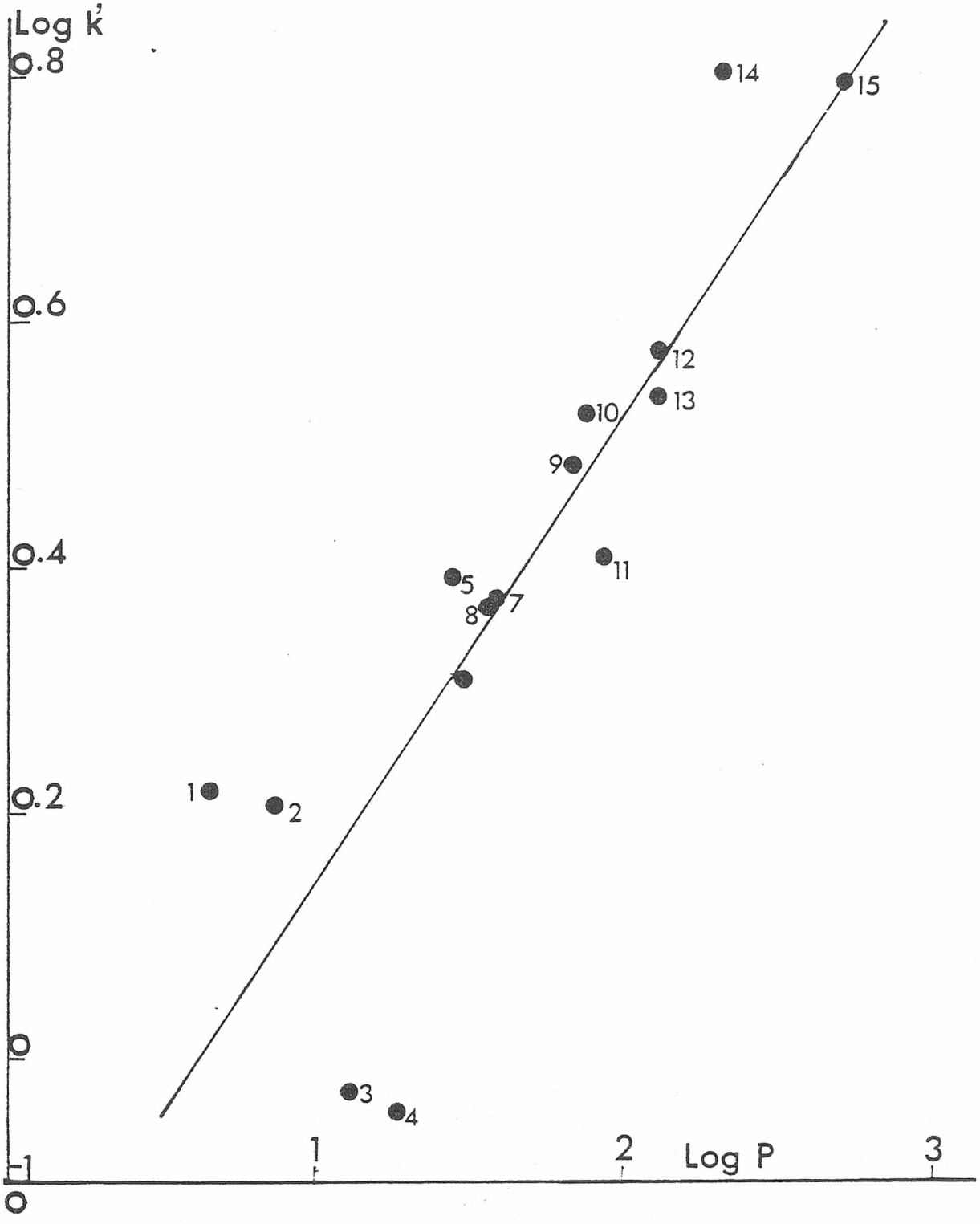


Figure 67A Correlations between log k' and π for several substituted benzenes. Chromatographic conditions: stationary phase Partisil ODS, mobile phase 30% v/v acetonitrile-water mixture. Compounds identification: 1- pyridine, 2- piperidine, 3- benzylalcohol, 4- salicylamide, 5- o-nitroaniline, 6- benzylaldehyde, 7- acetophenone, 8- p-chloroaniline, 9- p-chloroaniline, 10- nitrobenzene, 11- o-chloroaniline, 12- anisole, 13- benzene, 14- N,N-dimethylaniline, 15- toluene, (Ref. 287).

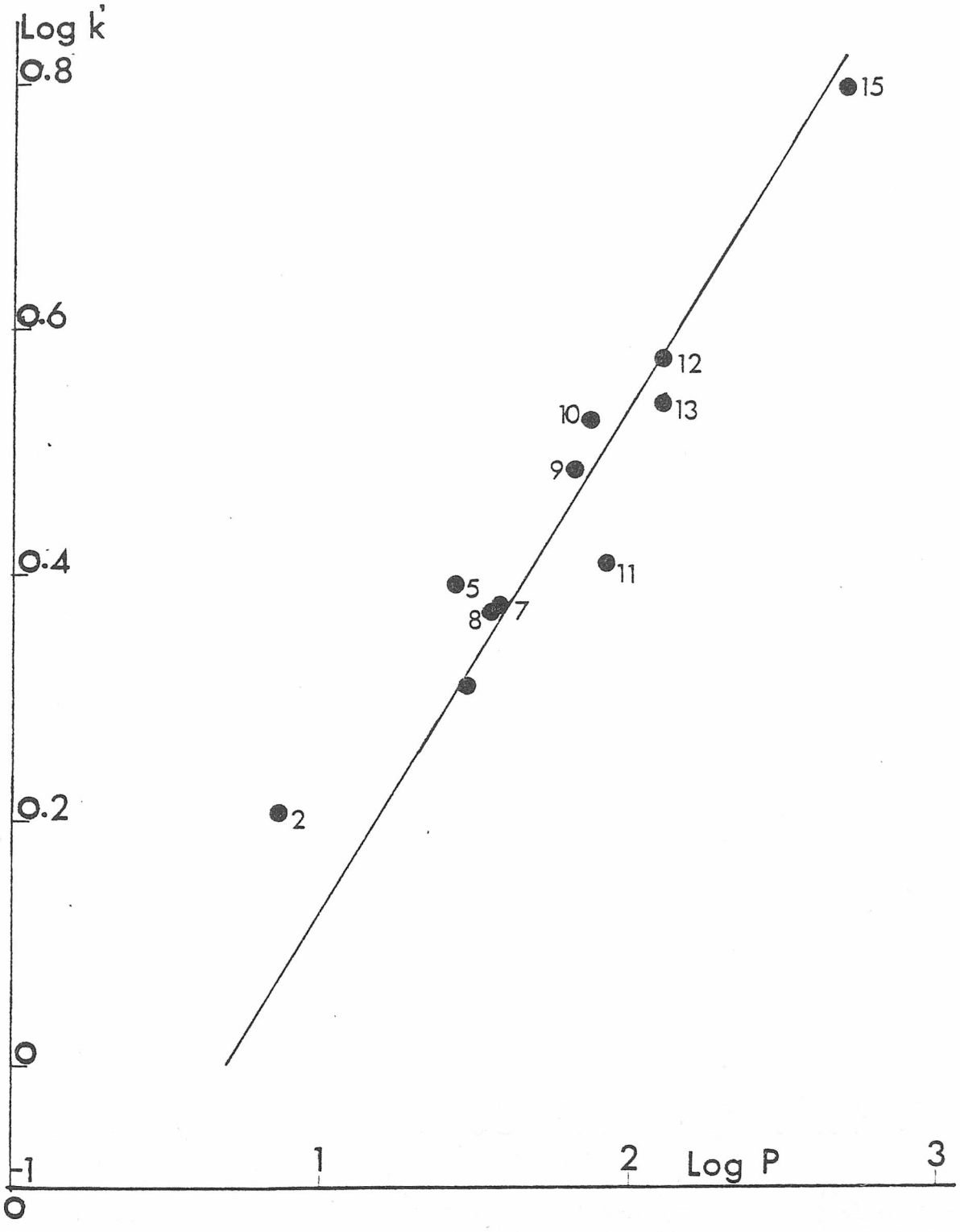


Figure 67B The same plot of Figure (67a) except compounds 1, 3, 4 and 14 are not included in the correlations. Compound identification as in Figure (67a).

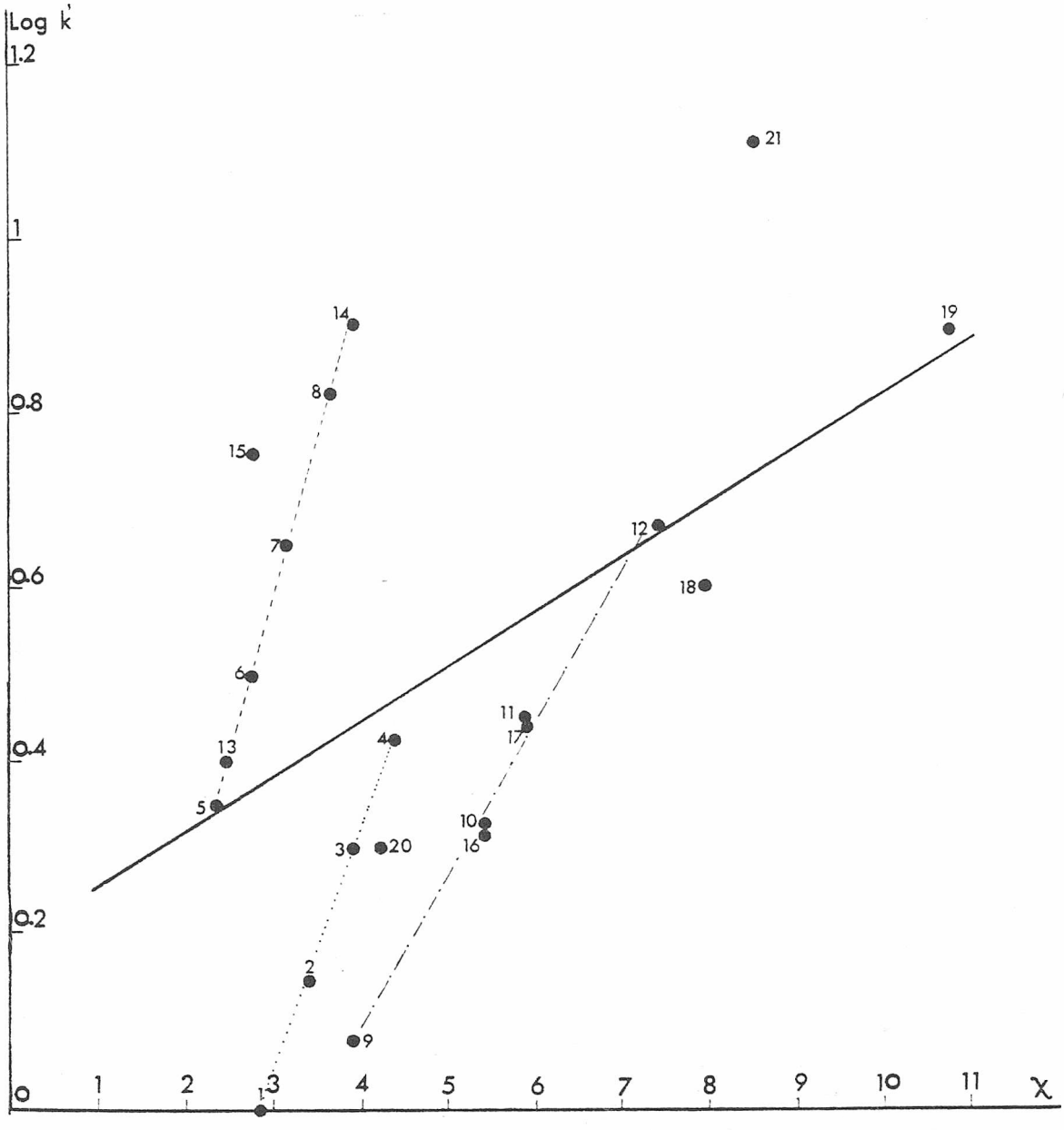


Figure 68 Correlations between log k' and χ for several aliphatic, aromatic and polynuclear hydrocarbon compounds. Chromatographic conditions: stationary phase C-8 Develosil, mobile phase 80% v/v methanol-water mixtures. Compounds identification: 1- toluene, 2- ethylbenzene, 3- n-propybenzene, 5- n-pentane, 6- n-hexane, 7- n-heptane, 8- n-octane, 9- naphthalene, 10- anthracene, 11- pyrene, 12- 3,4-benzpyrene, 13- cyclohexane, 14- trans-decahydronaphthalene, 15- adamantane, 16- diphenylmethane, 17- 1,2-diphenylethane, 18- triphenylmethane, 19- tetraphenylethylene, 20- fluorene, 21- bidiphenylethane, (Ref. 288).

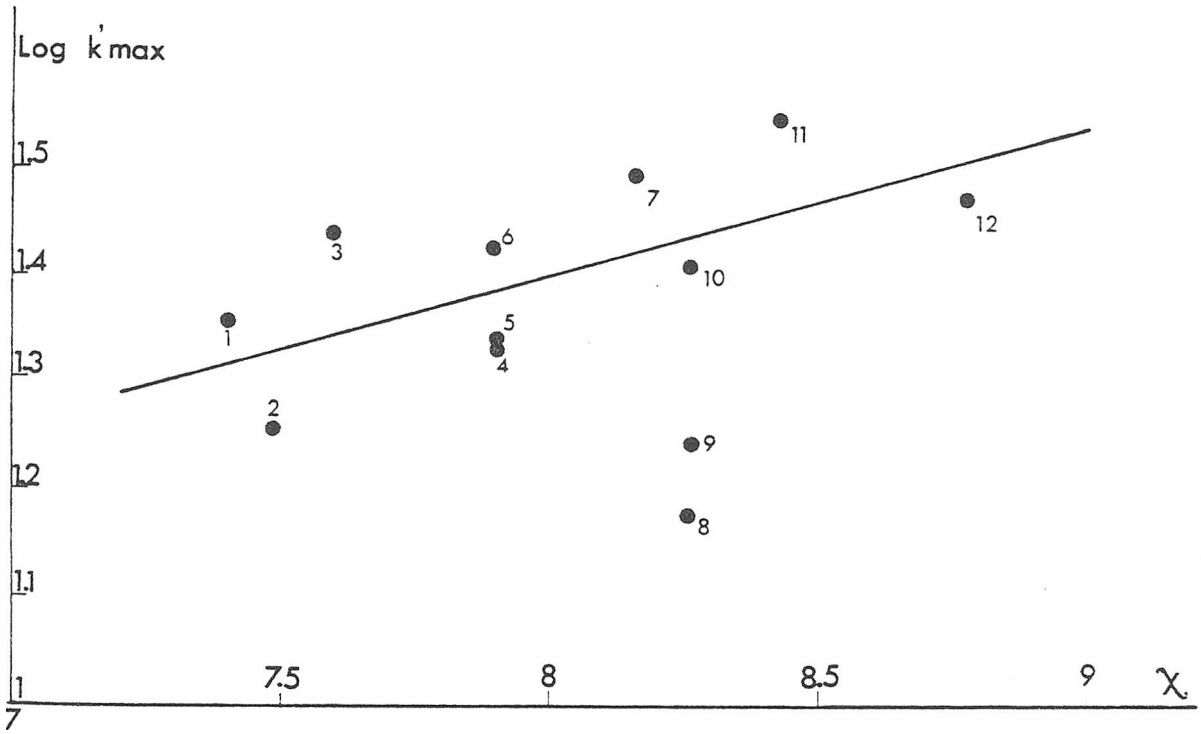


Figure 69 Correlations between log k' and $^{7}\chi$ for the twelve tricyclic antidepressants studied in Chapter (4) under ion-pairing conditions. Chromatographic conditions as in Figure (35). Compound identification: 1- desipramine, 2- mianserine, 3- nomifensine, 5- protriptyline, 6- nortriptyline, 7- maprotiline, 8- doxepin, 9- dothiepin, 10- amitriptyline, 11- clomipramine, 12- trimipramine.

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APPENDIX I Postgraduate courses

The following postgraduate courses and scientific meetings were attended in connection with this programme of research in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

(1) A residential course in HPLC at University of Sussex, June, 1980, sponsored by the Chemical Society of Great Britain.

(2) An undergraduate course for final Honours year students in advanced surface chemistry in the School of Chemistry, R.G.I.T.

(3) Symposium in HPLC, Feb. 1980, at University of Edinburgh, organised by the Chemical Society, G.B.

(4) Symposium in HPLC, Feb. 1981, at University of Edinburgh, organised by the Chemical Society, G.B.

(5) Continuous participation in meetings of Aberdeen HPLC users group throughout this research programme.

(6) Continuous personal review of the current literature.

APPENDIX II

Help file for the running of the computer programme HOOKE1

TY IIM.DAT

THIS IS AN INTERATIVE PROGRAM TO PRODUCE THE BEST LEAST SQUARE FIT OF THE EXPERIMENTAL DATA (CAPACITY FACTORS) VS (CONCENTRATION OF THE PAIRING ION ON THE C-18 SURFACE AND IN THE SOLVENTS). THE CONSTANTS K6 & K1 CAN THEN BE EVALUATED.
TO RUN THIS PROGRAM, A DATA FILE 'HOOKE.DAT' IS CREATED, FOLLOWING INFORMATIONS ARE THEN INPUT INTO THE FILE IN ORDER:
(1) NUMBER OF SETS OF DATA (M)
(2) CAPACITY FACTORS OF THE SOLUTE AT DIFFERENT CONCENTRATIONS OF PAING ION ON THE STATIONARY PHASE (G).
(3) CONCENTRATIONS OF THE PAIRING ION ON THE STATIONARY PHASE IN UMOL./GM (R)
(4) CONCENTRATION OF THE PAIRING ION IN THE MOBILE PHASE IN MMOL/LITRE (T).
(5) DESOLVATION CONSTANT OF THE SOLUTE (K2 IN THE IIM MODEL) , WHICH CAN BE CALCULATED WHEN (R)=Ø (Z1).
(6) SURFACE AREA OF A MOLECULE OF THE PAIRING ION IN ANSTRON SQUARE (A2).
(7) VALENCY OF THE COUNTER ION (C).
(8) TOTAL SURFACE AREA OF THE STATIONARY PHASE IN THE COLUMN IN METERE SQUARE (A1).
(9) WEIGHT OF THE STATIONARY PHASE IN THE COLUMN IN GRAM (Z5).
(1Ø) VOID VOLUME OF THE COLUMN IN CC (VM).
(11) BUFFER SALT CONC. IN MMOL/LITRE (B).
THE FORMAT OF INPUT IS 6G.
IN ORDER TO OBTAIN RELIABLE SOLUTION VECTORS, THIS PROGRAM HAS TO BE RUN OVER SEVERAL TIMES USING THE SAME SET OF DATA. DIFFERENT STEP SIZES AND INITIAL VALUES OF THE CONSTANTS K6 & K1 SHOULD BE INPUT EACH TIME UNTIL THE SAME SOLUTION VECTORS ARE OBTAINED.
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Listing of the programme HOOKE1

TY HOOKE1.FOR

```
00100   C     MODEL IS (A1-R*A2)((K6R)**(1/C)+Z1(S)**(1/C))/
00200   C     ((K1T)**(1/C)+(S)**(1/C))
00300         COMMON G,R,S,M,A1,A2,Z1,C,Z5,T
00400         DIMENSION IDEMH(14)
00500         DOUBLE PRECISION G(18),R(18),S(18),G2(18),G3(18),G4(18)
00600         DOUBLE PRECISION A4(18),T(18)
00700         DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
00800         DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
00900         WRITE(5,3)
01000   3     FORMAT(//,1H,'IF YOUU WANT HELP TYPE H, ?   ',$)
01100         READ(5,6)HELP
01200   6     FORMAT(A1)
01300         IF (HELP .EQ. 'H') GOTO 9
01400         GOTO 29
01500   9     OPEN (UNIT=1,FILE='IIM.DAT')
01600         DO 97 K5=1,32
01700         READ(1,99)(IDEMH(K3),K3=1,14)
01800   99    FORMAT(14A5)
01900   98    FORMAT(1H,/,14A5)
02000   97    WRITE(5,98)(IDEMH(K3),K3=1,14)
02100      29 OPEN (UNIT=1,FILE='HOOKE.DAT')
02200         READ(1,39)M,(G(I),I=1,M),(R(I),I=1,M),(T(I),I=1,M),Z1,A2,
02300        1C,A1,Z5,VM,B
02400      39 FORMAT(6G)
02500      59 N=2
02600   C***** CHANGE OF MAGNITUDES OF R & G ********
02700         DO 15 I=1,M
02800         G(I)=G(I)*VM/1000000
02900         R(I)=R(I)/1000000
03000      15 CONTINUE
03100      69 WRITE(5,47)
03200      47 FORMAT(/,1H,'INPUT STEP SIZES OF K6,K1,EPS, SEP BY RETURN.
')
03300         READ(5,20)(XK(I),I=1,N),EPS
03400      10 FORMAT(12G)
03500         WRITE(5,95)
03600      95 FORMAT(/,1H,'INPUT PARAMATER INITIAL VALUES OF K6,K1.')
03700         READ(5,20)(X0(K),K=1,N)
03800      20 FORMAT(G)
03900         DO 27 I=1,M
04000         S(I)=T(I)+B
04100     27  CONTINUE
04200   C**** CHANGE THE UNIT OF THE AREA OCCUPIED BY PAIRING ION.***
04300         A3=A2
04400         A2=A2*6.02*1000
04500   C**** COMPUTING PART********
04600         Y0=F(X0)
04700         DO 30 I=1,N
04800      30 X11(I)=X0(I)
04900         Y11=Y0
05000     111 DO 40 I=1,N
05100      40 X1(I)=X11(I)
```

```
05200          Y1=Y11
05300          I=0
05400      222 I=I+1
05500          DO 50 K=1,N
05600          IF(K.EQ.I) GOTO 60
05700          W=0.0D0; GOTO 70
05800       60 W=1.0D0
05900       70 X(K)=X1(K)+XK(K)*W
06000       50 CONTINUE
06100          Y=F(X)
06200          IF(Y.LT.Y1) GOTO 80
06300          DO 51 K=1,N
06400          IF(K.EQ.I) GOTO 61
06500          W=0.0D0; GOTO 71
06600       61 W=1.0D0
06700       71 X(K)=X1(K)-XK(K)*W
06800       51 CONTINUE
06900          Y=F(X)
07000          IF(Y.GE.Y1)GOTO 90
07100       80 DO 100 K=1,N
07200      100 X1(K)=X(K)
07300          Y1=Y
07400       90 IF(I.NE.N) GOTO 222
07500          IF(Y1.LT.Y0) GOTO 110
07600          DO 120 K=1,N
07700          IF(X11(K).NE.X0(K))GOTO 130
07800      120 CONTINUE
07900          DO 555 K=1,N
08000      555 XK(K)=XK(K)/10.0D0
08100          DO 666 K=1,N
08200          IF(DABS(XK(K)).GE.EPS)GOTO 111
08300      666 CONTINUE
08400          GOTO 333
08500      130 DO 140 K=1,N
08600      140 X11(K)=X0(K)
08700          Y11=Y0
08800          GOTO 111
08900     ****** COMPUTING FINISHED *****
09000      333 WRITE(5,150)
09100      150 FORMAT(1H ,'SOLUTION VECTOR-K6, THE ION-EXCHANGE CONSTANT'
)
09200          WRITE(5,160)X0(1)
09300      160 FORMAT(1H ,G)
09400          WRITE(5,155)
09500      155 FORMAT(1H ,'SOLUTION VECTOR-K1, THE ION-PAIRING CONSTANT')
09600          WRITE(5,160)X0(2)
09700          WRITE(5,660)Y0
09800      660 FORMAT(1H ,'FUNCTION VALUE=',G)
09900          WRITE(5,22)Z1
10000       22 FORMAT(///,1H,'K2=',G)
10100          WRITE(5,32)A3
10200       32 FORMAT(//,1H,'AP=',G)
10300          WRITE(5,576)A1
```

```
10400
10500     576  FORMAT(/,1H,'THE SURFACE AREA OF THE COLUMN =',G)
10600    C***** X0(1) IS K6 AND X0(2) IS K4 ********
10700          DO 4 I=1,M
10800          G2(I)=(A1-Z5*R(I)*A2)*(X0(1)*(R(I)**(1/C))+Z1*(S(I)**(1/C)
))
10900         1/(X0(2)*(T(I)**(1/C))+S(I)**(1/C))
11000          G3(I)=G(I)-G2(I)
11100          S1=S1+G3(I)
11200          S3=S3+(G3(I))**2
11300        4 CONTINUE
11400          S2=(S1/M)**2
11500          S4=((S3-MS2)/(M-1))**0.5
11600          DO 14 I=1,M
11700          G4(I)=G3(I)/S4
11800          A4(I)=A2*R(I)*Z5
11900       14 CONTINUE
12000          DO 25 I=1,M
12100          R(I)=R(I)*1000000
12200          G(I)=G(I)*1000000/VM
12300          G2(I)=G2(I)*1000000/VM
12400          G3(I)=G3(I)*1000000/VM
12500       25 CONTINUE
12600          WRITE(5,24)
12700       24 FORMAT(/,1H,6X,'R',10X,'GI',10X,'GC',10X,'GI-GC',6X,'AR. O
CP')
12800          DO 54 I=1,M
12900       44 FORMAT(5F12.5)
13000          WRITE(5,44)R(I),G(I),G2(I),G3(I),A4(I)
13100       54 CONTINUE
13200          GOTO 444
13300      110 DO 170 K=1,N
13400          X11(K)=2.0D0*X1(K)-X0(K)
13500      170 X0(K)=X1(K)
13600          Y0=Y1;Y11=F(X11)
13700          GOTO 111
13800      444 STOP;END
13900          DOUBLE PRECISION FUNCTION F(X)
14000          DOUBLE PRECISION X(10)
14100          DOUBLE PRECISION G(18),R(18),S(18),T(18)
14200          DOUBLE PRECISION SUM,TERM
14300          COMMON G,R,S,M,A1,A2,Z1,C,Z5,T
14400          SUM=0.0D0
14500    C************ X(1) IS K6, X(2) IS K4 **************
14600          DO 10 I=1,M
14700          TERM=G(I)-(A1-Z5*R(I)*A2)*(X(1)*(R(I)**(1/C))+Z1*S(I)**(1/
C))
14800         1/(X(2)*(T(I)**(1/C))+S(I)**(1/C))
14900          SUM=SUM+TERM*TERM
15000       10 CONTINUE
15100          F=SUM
15200          RETURN;END
@
```

Specimen data file and output for the programme HOOKE1 assuming the area occupied by the pairing ions on the C-18 surface is negligible.

```
TY HOOKE.DAT

13,22.35,74.12,93.82,108.82,152.94
181.18,202.65,203.533,185.29,155.29,131.76
115,97.65,0,.497,2.805,9.6098
14.2,20.22,22.33,26.641,31.874,43.53
49.477,51.1417,53.2825,0,.4757,2.3786
4.753,9.514,19.0294,23.79,33.3016,47.574
95.1474,142.7212,197.8458,274.7859,.000000157415,0
1,72.42,.5514,.51,2
@
 RUN HOOKE1


IF YOUU WANT HELP TYPE H, ?
INPUT STEP SIZES OF K6,K1,EPS, SEP BY RETURN.
.01
.01
.00001
INPUT PARAMETER INITIAL VALUES OF K6,K1.
1
1

SOLUTION VECTOR-K6, THE ION-EXCHANGE CONSTANT
  0.22311999999999991D+00
SOLUTION VECTOR-K1, THE ION-PAIRING CONSTANT
 -0.77799999999999999D+00
FUNCTION VALUE=  0.18321453656757868 2D-08


K2=  0.1574150E-06

AP=  0.0000000E+00
THE SURFACE AREA OF THE COLUMN =   72.42000
      R          GI          GC          GI-GC       AR. OCP
    0.00000    22.35000    22.35293    -0.00293     0.00000
    0.49700    74.12000    33.76018    40.35982     0.00000
    2.80500    93.82000    73.86940    19.95060     0.00000
    9.60980   108.82000   149.06457   -40.24457     0.00000
   14.20000   152.94000   171.99714   -19.05714     0.00000
   20.22000   181.18000   178.43923     2.74077     0.00000
   22.33000   202.65000   176.33530    26.31470     0.00000
   26.64100   203.53300   173.87095    29.66205     0.00000
   31.87400   185.29000   168.61056    16.67944     0.00000
   43.53000   155.29000   153.55855     1.73145     0.00000
   49.47700   131.76000   142.57539   -10.81539     0.00000
   51.14170   115.00000   132.56162   -17.56162     0.00000
   53.28250    97.65000   124.99712   -27.34712     0.00000
STOP

END OF EXECUTION
CPU TIME: 1.15  ELAPSED TIME: 48.32
EXIT
@
```

Specimen data file and output for the programme HOOKE1 taking into consideration the area occupied by the pairing ion on the C-18 surface not available for desolvation of solutes.

```
TY HOOKE.DAT

13,22.35,74.12,93.82,108.82,152.94
181.18,202.65,203.533,185.29,155.29,131.76
115,97.65,0,.497,2.805,9.6098
14.2,20.22,22.33,26.641,31.874,43.53
49.477,51.1417,53.2825,0,.4757,2.3786
4.753,9.514,19.0294,23.79,33.3016,47.574
95.1474,142.7212,197.8458,274.7859,.000000157415,36.29545
1,72.42,.5514,.51,2
@
 RUN HOOKE1



IF YOUU WANT HELP TYPE H, ?
INPUT STEP SIZES OF K6,K1,EPS, SEP BY RETURN.
.01
.01
.00001
INPUT PARAMETER INITIAL VALUES OF K6,K1.
1
1

SOLUTION VECTOR-K6, THE ION-EXCHANGE CONSTANT
  0.213249999999999991D+00
SOLUTION VECTOR-K1, THE ION-PAIRING CONSTANT
 -0.798919999999999999D+00
FUNCTION VALUE=  0.171943745086294643D-08


K2=  0.1574150E-06

AP=   36.29545
THE SURFACE AREA OF THE COLUMN =   72.42000
      R          GI          GC          GI-GC       AR. OCP
    0.00000    22.35000    22.35293    -0.00293     0.00000
    0.49700    74.12000    33.56034    40.55966     0.05988
    2.80500    93.82000    73.42205    20.39795     0.33795
    9.60980   108.82000   147.13202   -38.31202     1.15779
   14.20000   152.94000   171.50983   -18.56983     1.71082
   20.22000   181.18000   179.51837     1.66163     2.43611
   22.33000   202.65000   177.79884    24.85116     2.69032
   26.64100   203.53300   175.37320    28.15980     3.20971
   31.87400   185.29000   169.75145    15.53855     3.84018
   43.53000   155.29000   153.17674     2.11326     5.24450
   49.47700   131.76000   141.49235    -9.73235     5.96100
   51.14170   115.00000   131.72774   -16.72774     6.16156
   53.28250    97.65000   124.16610   -26.51610     6.41948
STOP

END OF EXECUTION
CPU TIME: 1.11  ELAPSED TIME: 41.93
EXIT
@
```

Help file for running of the computer programme HOOKE2.

TY IEDM.DAT

THIS IS AN INTERATIVE PROGRAM TO PRODUCE THE BEST LEAST SQUARE FIT OF EXPERIMENTAL DATA (CAPACITY FACTORS) VS (CONCENTRATION OF THE PAIRING ION ON THE STATIONARY PHASE) AND THE COMPOSITE CONSTANT (KDESKIE) CAN BE EVALUATED.
TO RUN THIS PROGRAM, A DATA FILE 'HOOKE.DAT' IS CREATED, FOLLOWING INFORMATIONS ARE THEN INPUT INTO THE FILE IN ORDER:
(1) NUMBER OF SETS OF DATA (M)
(2) CAPACITY FACTORS OF THE SOLUTE AT DIFFERENT CONCENTRATIONS OF PAING ION ON THE STATIONARY PHASE (G).
(3) CONCENTRATIONS OF THE PAIRING ION ON THE STATIONARY PHASE IN UMOL./GM (R)
(4) CONCENTRATIONS OF THE COUNTER ION (S) IN THE MOBILE PHASE IN MMOL/LITRE (S).
(5) DESOLVATION CONSTANT OF THE SOLUTE, KDES, WHICH CAN BE CALCULATED WHEN (R)=Ø (Z1).
(6) SURFACE AREA OF A MOLECULE OF THE PAIRING ION IN ANSTRON SQUARE (A2).
(7) VALENCY OF THE COUNTER ION (C).
(8) TOTAL SURFACE AREA OF THE STATIONARY PHASE IN THE COLUMN IN METERE SQUARE (A1).
(9) WEIGHT OF THE STATIONARY PHASE IN THE COLUMN IN GRAM (Z5).
(1Ø) VOID VOLUME OF THE COLUMN IN CC (VM).
THE FORMAT OF INPUT IS 6G.
IN ORDER TO OBTAIN AN RELIABLE SOLUTION VECTOR (COMPOSITE CONSTANT), THIS PROGRAM HAS TO BE RUN OVER SEVERAL TIMES ON THE SAME SET OF DATA. DIFFERENT STEP SIZE AND INITIAL VALUE OF THE COMPOSITE CONSTANT SHOULD BE INPUT EACH TIME UNTIL THE SAME SOLUTION VECTOR IS OBTAINED.
****

@

Listing of the programme HOOKE2.

TY HOOKE2.FOR

```
00100   C      MODEL IS G=Z1(A1-R*A2)+K2(R/S)**(1/C)(A1-RA2)
00200          COMMON G,R,S,M,A1,A2,Z1,C,Z5
00300          DIMENSION IDEMH(14)
00400          DOUBLE PRECISION G(18),R(18),S(18),G2(18),G3(18),G4(18)
00500          DOUBLE PRECISION A4(18)
00600          DOUBLE PRECISION EPS,XK,X0,Y0,X11,Y11,X1,Y1,X,Y,F,W
00700          DIMENSION X0(10),X(10),X11(10),X1(10),XK(10)
00800          WRITE(5,3)
00900   3      FORMAT(//,1H,'IF YOUU WANT HELP TYPE H, ?   ',$)
01000          READ(5,6)HELP
01100   6      FORMAT(A1)
01200          IF (HELP .EQ. 'H') GOTO 9
01300          GOTO 29
01400   9      OPEN (UNIT=1,FILE='IEDM.DAT')
01500          DO 97 K5=1,31
01600          READ(1,99)(IDEMH(K3),K3=1,14)
01700   99     FORMAT(14A5)
01800   98     FORMAT(1H,/,14A5)
01900   97     WRITE(5,98)(IDEMH(K3),K3=1,14)
02000      29  OPEN (UNIT=1,FILE='HOOKE.DAT')
02100          READ(1,39)M,(G(I),I=1,M),(R(I),I=1,M),(S(I),I=1,M),Z1,A2,
02200        1C,A1,Z5,VM
02300      39  FORMAT(6G)
02400      59  N=1
02500   C***** CHANGE OF MAGNITUDES OF R & G ********
02600          DO 15 I=1,M
02700          G(I)=G(I)*VM/1000000
02800          R(I)=R(I)/1000000
02900      15  CONTINUE
03000      69  WRITE(5,47)
03100      47  FORMAT(/,1H,'INPUT STEP SIZE OF KDESKIE,EPS, SEP BY RETURN
.')
03200          READ(5,20)(XK(I),I=1,N),EPS
03300      10  FORMAT(12G)
03400          WRITE(5,95)
03500      95  FORMAT(/,1H,'INPUT PARAMETER INITIAL VALUES OF KDESKIE.')
03600          READ(5,20)(X0(K),K=1,N)
03700      20  FORMAT(G)
03800   C**** CHANGE THE UNIT OF THE AREA OCCUPIED BY PAIRING ION.***
03900          A3=A2
04000          A2=A2*6.02*1000
04100   C**** COMPUTING PART********
04200          Y0=F(X0)
04300          DO 30 I=1,N
04400      30  X11(I)=X0(I)
04500          Y11=Y0
04600     111  DO 40 I=1,N
04700      40  X1(I)=X11(I)
04800          Y1=Y11
04900          I=0
05000     222  I=I+1
05100          DO 50 K=1,N
```

```
05200          IF(K.EQ.I) GOTO 60
05300          W=0.0D0; GOTO 70
05400       60 W=1.0D0
05500       70 X(K)=X1(K)+XK(K)*W
05600       50 CONTINUE
05700          Y=F(X)
05800          IF(Y.LT.Y1) GOTO 80
05900          DO 51 K=1,N
06000          IF(K.EQ.I) GOTO 61
06100          W=0.0D0; GOTO 71
06200       61 W=1.0D0
06300       71 X(K)=X1(K)-XK(K)*W
06400       51 CONTINUE
06500          Y=F(X)
06600          IF(Y.GE.Y1)GOTO 90
06700       80 DO 100 K=1,N
06800      100 X1(K)=X(K)
06900          Y1=Y
07000       90 IF(I.NE.N) GOTO 222
07100          IF(Y1.LT.Y0) GOTO 110
07200          DO 120 K=1,N
07300          IF(X11(K).NE.X0(K))GOTO 130
07400      120 CONTINUE
07500          DO 555 K=1,N
07600      555 XK(K)=XK(K)/10.0D0
07700          DO 666 K=1,N
07800          IF(DABS(XK(K)).GE.EPS)GOTO 111
07900      666 CONTINUE
08000          GOTO 333
08100      130 DO 140 K=1,N
08200      140 X11(K)=X0(K)
08300          Y11=Y0
08400          GOTO 111
08500     C****** COMPUTING FINISHED *****
08600      333 WRITE(5,150)
08700      150 FORMAT(1H ,'SOLUTION VECTOR-KDESKIE')
08800          WRITE(5,160)X0(1)
08900      160 FORMAT(1H ,G)
09000          WRITE(5,660)Y0
09100      660 FORMAT(1H ,'FUNCTION VALUE=',G)
09200          WRITE(5,22)Z1
09300       22 FORMAT(///,1H,'KDES=',G)
09400          WRITE(5,32)A3
09500       32 FORMAT(//,1H,'AP=',G)
09600          WRITE(5,576)A1
09700
09800      576  FORMAT(/,1H,'THE SURFACE AREA OF THE COLUMN IN M**2 =  ',G
)
09900     C****** X0(1) IS KDESKIE ******
10000          DO 4 I=1,M
10100          G2(I)=Z1*(A1-Z5*R(I)*A2)+X0(1)*((R(I)/S(I))**(1/C))*
10200         1(A1-Z5*A2*R(I))
10300          G3(I)=G(I)-G2(I)
```

```
10400          S1=S1+G3(I)
10500          S3=S3+(G3(I))**2
10600        4 CONTINUE
10700          S2=(S1/M)**2
10800          S4=((S3-MS2)/(M-1))**0.5
10900          DO 14 I=1,M
11000          G4(I)=G3(I)/S4
11100          A4(I)=A2*R(I)*Z5
11200       14 CONTINUE
11300          DO 25 I=1,M
11400          R(I)=R(I)*1000000
11500          G(I)=G(I)*1000000/VM
11600          G2(I)=G2(I)*1000000/VM
11700          G3(I)=G3(I)*1000000/VM
11800       25 CONTINUE
11900          WRITE(5,24)
12000       24 FORMAT(/,1H,6X,'R',10X,'GI',10X,'GC',10X,'GI-GC',6X,'AR. O
CP')
12100          DO 54 I=1,M
12200       44 FORMAT(5F12.5)
12300          WRITE(5,44)R(I),G(I),G2(I),G3(I),A4(I)
12400       54 CONTINUE
12500          GOTO 444
12600      110 DO 170 K=1,N
12700          X11(K)=2.0D0*X1(K)-X0(K)
12800      170 X0(K)=X1(K)
12900          Y0=Y1;Y11=F(X11)
13000          GOTO 111
13100      444 STOP;END
13200          DOUBLE PRECISION FUNCTION F(X)
13300          DOUBLE PRECISION X(10)
13400          DOUBLE PRECISION G(18),R(18),S(18)
13500          DOUBLE PRECISION SUM,TERM
13600          COMMON G,R,S,M,A1,A2,Z1,C,Z5
13700          SUM=0.0D0
13800    C************* X(1) IS KDESKIE ******
13900          DO 10 I=1,M
14000          TERM=G(I)-Z1*(A1-A2*Z5*R(I))-X(1)*((R(I)/S(I))**(1/C))
14100         1*(A1-A2*Z5*R(I))
14200          SUM=SUM+TERM*TERM
14300       10 CONTINUE
14400          F=SUM
14500          RETURN;END
@
```

Specimen data file and output for the programme HOOKE2.

```
TY HOOKE.DAT


13,22.35,74.12,93.82,108.82,152.94
181.18,202.65,203.533,185.29,155.29,131.76
115,97.65,0,.497,2.805,9.6098
14.2,20.22,22.33,26.641,31.874,43.53
49.477,51.1417,53.2825,2,2.4757,4.3786
6.753,11.514,21.0294,25.799,35.3016,49.574
97.1474,144.7212,199.8458,276.7859,.000000157415,36.29545
1,72.42,.5514,.51
@

 RUN HOOKE2


IF YOUU WANT HELP TYPE H, ?
INPUT STEP SIZE OF KDESKIE,EPS, SEP BY RETURN.
.001
.00000001
INPUT PARAMETER INITIAL VALUES OF KDESKIE.
1

SOLUTION VECTOR-KDESKIE
  0.104902125000000000D+01
FUNCTION VALUE=  0.126072720908344330D-07


KDES=  0.1574150E-06

AP=   36.29545
THE SURFACE AREA OF THE COLUMN IN M**2 =     72.42000
      R          GI          GC          GI-GC      AR. OCP
    0.00000    22.35000    22.35293    -0.00293     0.00000
    0.49700    74.12000    52.21384    21.90616     0.05988
    2.80500    93.82000   117.23008   -23.41008     0.33795
    9.60980   108.82000   230.58438  -121.76438     1.15779
   14.20000   152.94000   201.19580   -48.25580     1.71082
   20.22000   181.18000   160.01069    21.16931     2.43611
   22.33000   202.65000   145.66423    56.98577     2.69032
   26.64100   203.53300   128.79602    74.73698     3.20971
   31.87400   185.29000   111.86465    73.42535     3.84018
   43.53000   155.29000    82.64727    72.64273     5.24450
   49.47700   131.76000    67.24769    64.51231     5.96100
   51.14170   115.00000    55.32783    59.67217     6.16156
   53.28250    97.65000    46.50528    51.14472     6.41948
STOP

END OF EXECUTION
CPU TIME: 0.45  ELAPSED TIME: 34.96
EXIT
@
```

APPENDIX III

COMMUNICATIONS AND PUBLICATIONS

C. T. Hung and R. B. Taylor
Mechanism of Retention of Acidic Solutes by Octadecyl Silica using Quaternary Ammonium Pairing Ions as Ion Exchangers
J. Chromatogr., 202 (1980), 333-345.

C. T. Hung and R. B. Taylor
Ion-Exchange Desolvation Mechanism on Octadecyl Silica using Anionic Hydrophobic Pairing Ions
J. Chromatogr., 209 (1981), 175-190.

R. B. Taylor and C. T. Hung
An Ion Pairing HPLC Investigation of the Acidity produced in Thermally Degraded Dextrose Solution
J. Pharm. Pharmac. Suppl., 33, (1981), 101P.

C. T. Hung, A. B. Selkirk and R. B. Taylor
A Chromatographic Quality Control Procedure based on HPLC for 5-Hydroxymethylfurfural in Autoclaved D-Glucose Infusion Fluids
J. Hospital and Clinical Pharmacy, 7 (1982), 17.

C. T. Hung, R. B. Taylor and N. Paterson
Investigation of the Effect of Added Organic Amines on the Chromatography of Tricyclic Antidepressant Drugs using Reverse Phase Chromatography on Octadecyl Silica with Sodium Laurylsulphate as Pairing Ion.
J. Chromatogr., 240 (1982) 61-73.

D. G. Durham, C. T. Hung and R. B. Taylor
Identification of the Acids Produced during Autoclaving of D-Glucose Solutions using HPLC.
International J. Pharmaceutics (in the Press).

Reprinted from

Journal of Chromatography, 202 (1980) 333–345
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,290

MECHANISM OF RETENTION OF ACIDIC SOLUTES BY OCTADECYL SILICA USING QUATERNARY AMMONIUM PAIRING IONS AS ION EXCHANGERS

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(First received June 27th, 1980; revised manuscript received August 27th, 1980)

SUMMARY

The adsorption isotherms of four commonly used quaternary ammonium pairing ions in aqueous solution on ODS Hypersil are reported. The capacity factors of four substituted benzoic acids as test solutes are measured as a function of pairing ion concentration under conditions of complete solute ionisation. The chromatographic behaviour of an undissociated solute, 5-hydroxymethylfurfuraldehyde, is also examined under these conditions. Over the wide range of pairing-ion concentrations used, maximum values of capacity factors are observed.

A mechanism of retention, combining desolvation and ion exchange, which considers the effect of adsorbed pairing ion on surface available for desolvation is proposed. On the basis of this mechanism an equation relating capacity factor and adsorbed pairing-ion concentration is derived. The data obtained in this investigation are fitted to the equation and the desolvation and ion-exchange constants are estimated. The behaviour of the neutral solute 5-hydroxymethylfurfuraldehyde is examined in the light of the derived equation.

INTRODUCTION

Although ionised solute species may be chromatographed using alkyl-modified silica with aqueous solvents[1], the addition of low concentrations of quaternary amines to the mobile phase has been shown to improve separation of acidic solutes[2,3]. Corresponding improvement in the chromatography of basic solutes has been obtained by the addition of alkyl sulphonates[4].

The mechanism of retention of such ionised solutes has been interpreted[5–9] on the basis of ion pairs, formed between the ionised solute and the added pairing ion, being adsorbed by the hydrophobic stationary phase. Following evidence that the pairing ion, originally in the aqueous mobile phase, is adsorbed by the stationary phase, an alternative mechanism based on the *in situ* formation of an ion exchange matrix was proposed[10]. Several workers have demonstrated the phenomenon of

0021-9673/80/0000–0000/$02.25

pairing-ion adsorption on a quantitative basis, fitting results obtained to various isotherm types[2,8,11,12].

Quantitative evidence for the ion-pairing mechanism has been based largely on the linear dependence of solute capacity factor with mobile phase pairing-ion concentration[6,8,13]. Recent work taking into account competitive adsorption of the pairing ion, although producing an alternative dependence, has supported this mechanism[14].

That adsorption of pairing ion occurs on chemically bonded reversed phases has been taken as evidence of an ion-exchange mechanism being operative. Quantitative relationships between solute capacity factors or distribution coefficients and mobile phase pairing-ion concentration have been established[2,11] using an appropriate isotherm. In addition the inverse dependence of capacity factors on counter-ion concentration[15] has further supported the ion exchange hypothesis. Only recently, however, has capacity factor dependence on experimentally determined adsorbed pairing-ion and mobile phase counter-ion concentrations been demonstrated[16].

The ion-pairing concept also differs from that of ion exchange in that it indicates that desolvation of the ion pair is more readily achieved than that of the ionised solute alone. The ion-exchange mechanism takes no account of the hydrophobicity of the solute but implies that separation is based on differences in the ion-exchange constants of various solutes.

It has been shown by several workers[2,5,9,17,18] that, over wide ranges in mobile phase pairing-ion concentration, the dependence of capacity factors on pairing-ion concentration is complex. It may reach a plateau or indeed pass through a maximum. This would indicate that the processes occurring during such chromatographic separations are considerably more complex than those described by either the ion-pairing or ion-exchange approaches.

It is the purpose of the present work to determine the adsorption behaviour of several of the more commonly used quaternary ammonium alkyl pairing ions on ODS Hypersil as a typical capped C_{18} stationary phase. Using various benzoic acids as test solutes, it is intended to examine the relationships between capacity factors and pairing-ion concentration both in the mobile and stationary phases. It is intended to use sufficiently wide concentration ranges to observe the reported decrease of capacity factor and to relate this to the coverage of the adsorbent surface by pairing ion.

EXPERIMENTAL

Apparatus

Chromatographic column loading and column desorption were carried out using an Applied Chromatography Systems 750/3 dual piston pump and a Pye Unicam LC-3 variable wavelength UV detector. Injection for chromatography was via a Rheodyne 7125 valve. Chromatographic columns were usually 50 × 5 mm I.D. slurry packed although, for tetrabutylammonium sulphate, a very short 4 × 5 mm I.D. column was made. Columns for isotherm measurements were 250 × 5 mm I.D. All measurements were made at ambient temperature.

Materials

Tetramethylammonium sulphate (TMA), tetraethylammonium bromide (TEA) tetrabutylammonium sulphate (TBA), 2,4-dimethylbenzoic acid (2,4DMBA), naphthoic acid (NA) and 4-ethylbenzoic acid (4EBA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). Cetrimide (CTAB) was obtained from I.C.I. Pharmaceuticals (Macclesfield, Great Britain) and benzoic acid (BA) from Koch-Light Labs. (Colnbrook, Great Britain). 5-Hydroxymethylfurfuraldehyde (5HMF) was obtained from Sigma (St. Louis, MO, U.S.A.). Water was double-distilled in glass and all other reagents were of Analar or comparable quality. ODS Hypersil was purchased from Shandon (London, Great Britain).

Procedures

The adsorption isotherms for TMA, TEA, TBA and CTAB were determined by pumping the appropriate concentrations of pairing ion in water through the column until the absorbance, measured at a suitable low wavelength, was constant with time. Although the absorbance was not linear with concentration, the equilibrium state could be estimated by this procedure. Ethanol was used to desorb the pairing ion until the absorbance of the eluent was again constant, and the total eluent was collected. The mass of the pairing salt deloaded was determined after evaporating the solvent under reduced pressure and was corrected for the dead volume of the system. The deloading process usually required some 200 column volumes and twice this volume was passed. At the lowest concentrations of TMA employed, the coefficient of variation measured on four replicates was 16%.

Chromatographic measurements

A standard eluent of 0.002 *M* disodium hydrogen phosphate was employed at pH 7.4 containing the appropriate concentration of pairing salt. The column was equilibrated to this concentration before chromatographic results were recorded. The four benzoic acids used as solutes were chromatographed at a concentration of 0.01 *M* and a detection wavelength of 240 nm. 5HMF was injected at a concentration of 0.005 *M* and monitored at the same wavelength.

RESULTS AND DISCUSSION

Isotherms

The isotherms obtained for the various pairing ions are shown in Fig. 1. The curves obtained for the symmetrical tetraalkyl pairing ions resemble Type L according to the Giles classification[19]. This form is taken to indicate gradual surface coverage of an adsorbent, terminating in a plateau region where the amount adsorbed does not vary with the solution concentration. At low concentrations this isotherm will approximate to a Type C or linear isotherm, with the amount adsorbed directly proportional to the solution concentration. The surfactant pairing ion (CTAB) appears quite different and is markedly H Type. This indicates high adsorption even at very low solution concentration with no proportional region present. This is consistent with previously reported work where the time required for equilibration of a C_{18} phase with low concentrations of cetrimide has been emphasised[18]. It would

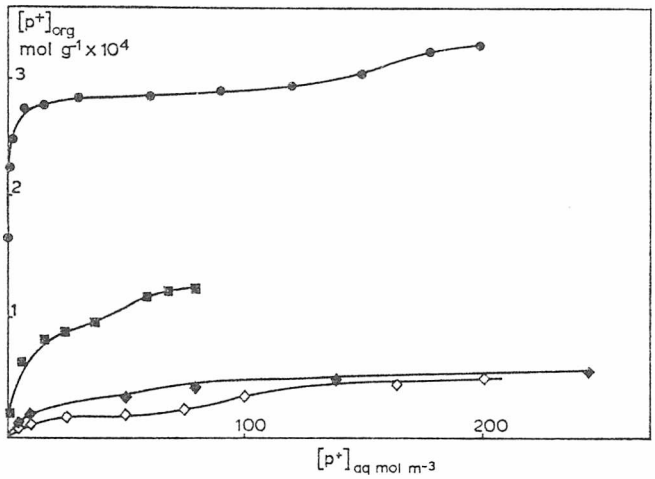

Fig. 1. Adsorption isotherms for various quaternary ammonium pairing ions for the ODS Hypersil–water system. ◊, TMA; ◆, TEA; ■, TBA; ●, CTAB.

appear from the present results that regulation of the surface concentration of very hydrophobic pairing ions would be very critical in aqueous solutions.

Although the results in Fig. 1 are represented as isotherms, it is obvious that each pairing ion used has a different plateau in terms of amount adsorbed. Indeed the larger the pairing ion, the more surface there appears to be available. This limits the usefulness of the isotherm as a general method of describing the adsorption behaviour of different pairing ions on the C_{18} surface. No attempt therefore is made to fit the curves to Freundlich or Langmuir equations. The adsorption data are used directly in subsequent sections to estimate the area of the C_{18} surface covered by a given pairing ion at appropriate aqueous concentrations. A relevant property of the pairing ion with respect to its adsorption would appear to be the hydrophobicity as measured by some characteristic parameter of that part of the pairing ion in contact with the C_{18} surface. A reasonable choice is the hydrophobicity of the longest hydrocarbon chain of the pairing ion. Correlation of the surface concentration at 0.05 *M* aqueous phase concentration with the Hansch π parameter[20] produces a regression line

$$\text{Amount adsorbed } (\mu\text{mol g}^{-1}) = 46.5\,\pi - 18 \qquad R^2 = 0.991$$

which indicates a good proportional relationship at a plateau region on the isotherms.

These results in aqueous solution show that the commonly used quaternary ammonium ions are indeed adsorbed to varying extents on the C_{18} silica and that the extent depends more upon the nature of the pairing ion than upon the aqueous-phase concentration. It is a consequence of this that such adsorbed species may act as *in situ* ion exchangers for other solutes and also that, depending on the nature and concentration of adsorbed pairing ion, some of the C_{18} surface will be occupied and thus be unavailable for the desolvation of solutes during chromatography. This latter aspect is considered further below, in the interpretation of the capacity factor variations for charged and uncharged solutes with pairing ion type and concentration.

Theoretical

The retention of an ionised solute in the presence of pairing ion on a C_{18} reversed-phase system may be considered as occurring by two separate mechanisms. One is the desolvation of the solute on the C_{18} surface. This may be minimal in the case of a highly ionised solute resulting in short retention times but may be appreciable if the solute is hydrophobic even when ionised.

The nett retention volume is given by

$$V_{r1} - V_m = K_1 A'_s \tag{1}$$

where V_{r1} is the measured retention volume, V_m the volume of mobile phase in the column, A'_s the surface area of the stationary phase available for desolvation and K_1 the desolvation constant or partition coefficient.

The other mechanism suggested is by an ion-exchange equilibrium of the form

$$(P_n{}^+C^{n-})_{org} + nA^-{}_{aq} \rightleftharpoons n(P^+A^-)_{org} + C^{n-}{}_{aq}$$

$(P_n{}^+C^{n-})_{org}$ refers to a monovalent pairing ion together with the possibly multivalent (n) counter ion adsorbed to the C_{18} surface, and A^- a monovalent anion as solute.

An ion-exchange constant can be written as

$$K_{IE} = \frac{[P^+A^-]^n_{org}\,[C^{n-}]_{aq}}{[P^+_nC^{n-}]_{org}\,[A^-]^n_{aq}} \tag{2}$$

From which, if the distribution for A^- between organic and aqueous phases is written as

$$D_{A^-} = \frac{[P^+A^-]_{org}}{[A^-]_{aq}} \tag{3}$$

by substitution for $[P^+A^-]_{org}$, we have

$$D_{A^-} = \left(\frac{K_{IE}\,[P^+_nC^{n-}]_{org}}{[C^{n-}]_{aq}}\right)^{1/n} \tag{4}$$

Such a relationship alone does not allow for any desolvation of the ion pair formed at the C_{18} surface due to the hydrophobicity of the solute. It does not account for differences in distribution, and thus retention, among different solutes other than on purely electrostatic terms. To explain the variation in retention, it is suggested that an ion-exchange reaction occurs between the ionised solute and the adsorbed pairing ion followed by desolvation of the neutralised solute on to the C_{18} surface. This desolvation will be proportional to the hydrophobicity of the solute as described by a desolvation constant K_2, which will act to increase the electrostatic effect of ion exchange. K_2, although analogous to K_1, may be of a different magnitude since it refers to a solute with no nett ionic charge. The retention of the bound species in on exchange will also be proportional to the area of the stationary phase available for desolvation, so that the nett retention volume for a solute retained by an ion-exchange–desolvation mechanism is given by

$$V_{r2} - V_m = K_2 A'_s \left(\frac{K_{IE}\,[P^+_nC^{n-}]_{org}}{[C^{n-}]_{aq}}\right)^{1/n} \tag{5}$$

The total nett retention volume ($V_{rT} - V_m$) is given by

$$V_{rT} - V_m = K_1 A_s' + K_2 A_s' \left(\frac{K_{IE} [P_n^+ C^{n-}]_{org}}{[C^{n-}]_{aq}} \right)^{1/n} \quad (6)$$

and the column capacity factor k' by

$$k' = \frac{1}{V_m} \left\{ K_1 A_s' + K_2 A_s' \left(\frac{K_{IE} [P_n^+ C^{n-}]_{org}}{[C^{n-}]_{aq}} \right)^{1/n} \right\} \quad (7)$$

A'_s is related to the total area of the C_{18} stationary phase, A_s, by the relationship

$$A_s' = A_s - [P_n^+ C^{n-}]_{org} A_P \quad (8)$$

where A_P is the area per mole occupied by a particular adsorbed pairing ion. A_s' is difficult to define but can be thought of as the effective area available for adsorption of any species in terms of C_{18} surface.

Substituting A_s' in the expression for k' we obtain

$$k' = \frac{1}{V_m} (A_s - [P_n^+ C^{n-}]_{org} A_P) \left\{ K_1 + K_2 \left(\frac{K_{IE} [P_n^+ C^{n-}]_{org}}{[C^{n-}]_{aq}} \right)^{1/n} \right\} \quad (9)$$

This equation relates the capacity factor of a given solute to the adsorbed pairing-ion concentration and mobile phase counter-ion concentration, taking into account the effective area of C_{18} available for desolvation. It shows a much more complex dependence of k' on adsorbed pairing ion concentration than has hitherto been suggested.

The form of the dependence of k' on adsorbed pairing-ion concentration can be seen more readily if the situation of monovalent counter ion only is considered. The equation can be rearranged after setting n to unity as

$$k' = \frac{1}{V_m} (A_s K_1 - K_1 [P^+ C^-]_{org} A_P + K_2 K_{IE} A_s \frac{[P^+ C^-]_{org}}{[C^-]_{aq}} - K_2 K_{IE} A_P \frac{[P^+ C^-]_{org}^2}{[C^-]_{aq}}) \quad (10)$$

k' is thus expressed as a quadratic in $[P^+ C^-]_{org}$ which exhibits a maximum value. This is in agreement with the results obtained by several workers when considering the capacity factor dependence on aqueous pairing-ion concentration. Following the schematic representation of the mechanism of retention in solvophobic chromatography[21] and ionic interaction chromatography[22], the two processes treated above are diagrammatically represented in Fig. 2.

Chromatographic results

The dotted curves in Figs. 3(a–d) represent the variation in k' for the four acidic solutes with aqueous or mobile phase concentrations of the various pairing ions. For the tetramethyl, tetraethyl and tetrabutyl compounds, clearly observed maxima are evident. The maxima are more pronounced the greater the hydrophobicity

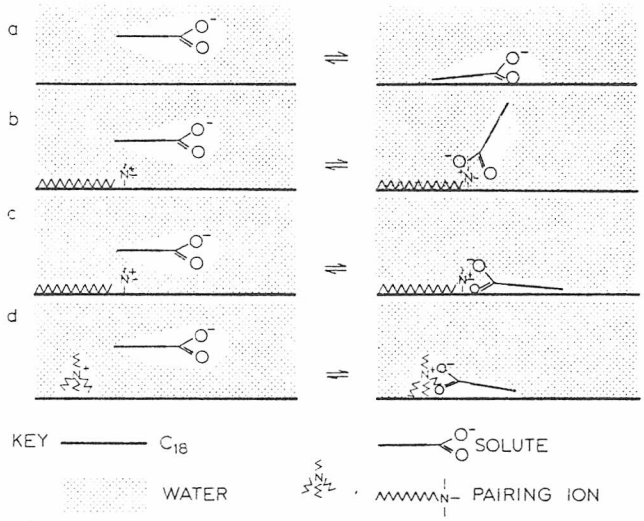

Fig. 2. Schematic representation of the postulated retention mechanisms discussed in the text. (a) Desolvation of an ionised solute; (b) ion exchange involving an adsorbed pairing ion without subsequent desolvation of solute; (c) and (d) ion exchange reinforcing desolvation of solute for adsorbed surfactant (c) and non surfactant (d) pairing ions.

of the solute and the pairing ion. The k' values become very large, reflecting the marked ion-exchange–solvophobic retention effect in wholly aqueous eluent. In the case of cetrimide, the only experimentally accessible k' values occur on the decreasing portion of the curve. This is interpreted as a consequence of the H-type isotherm obtained for this compound whereby, even at extremely low concentrations, appreciable concentrations of adsorbed pairing ion exist.

The increase of k' with $[P_n{}^+C^{n-}]_{aq}$ has previously been taken to support both the ion exchange[23] and the ion-pairing[24] interpretation of retention. Neither of these mechanisms, however, can account for the general decrease of k' observed in this and previous investigations where high pairing-ion concentrations were used. The decrease in k' has been attributed to micelle formation in the case of cetrimide by previous workers. In the present investigation, however, the dercease is seen to be general even for the non-surface-active pairing ions. The theoretical treatment of the model described above predicts this decrease when taken in conjunction with the experimentally measured adsorbed pairing ion concentrations, $[P_n{}^+C^{n-}]_{org}$. In eqns. 9 and 10, k' may decrease owing to two quite dissimilar effects. The C_{18} surface area available for desolvation subsequent to ion exchange will decrease according to the term $A_s - [P_n{}^+C^{n-}]_{org}A_P$. Also k' will decrease as $[C^{n-}]$ increases as a result of added pairing salt. Under the experimental conditions chosen, using low ionic strength buffer to minimise purely hydrophobic chromatography, it is not possible to separate these two effects, *i.e.* increasing $[P^+C^{n-}]_{aq}$ will tend to increase $[P_n{}^+C^{n-}]_{org}$ according to the isotherm, thus decreasing available surface, and will also increase $[C^{n-}]_{aq}$ directly.

In order to test quantitatively the fit of the derived equations (9 and 10) with the experimental points, the following procedure was adopted. The value of A_s for

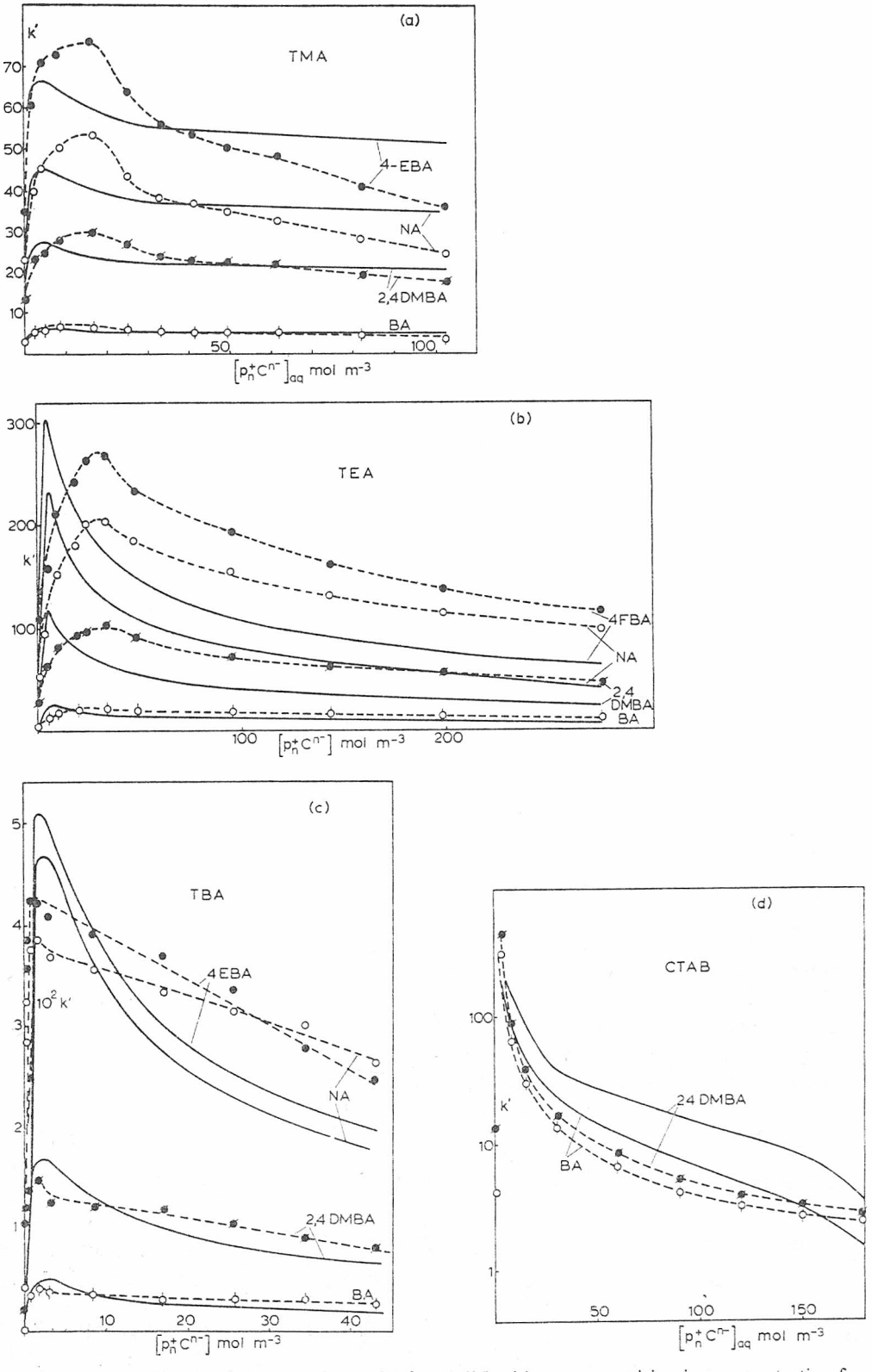

Fig. 3. Plots showing the variation of capacity factor (k') with aqueous pairing-ion concentration for the four acidic solutes and various pairing ions (a–d). Dashed lines represent experimental results. Full lines represent calculated curves.

the 5-μm silica ODS was taken[25] as 142 m^2 cm^{-3}. The factor A_P was calculated on the basis of accepted atomic dimensions, bond angles and lengths for each of the pairing ions in the fully extended conformation using data and calculations contained in the CAMSEQ software system[26]. The areas of the symmetrical pairing ions were calculated as circles with alkyl chain lengths as radius, and that of cetrimide as the molecule in a flat position on the adsorbent surface. For various aqueous concentrations of pairing ion, the corresponding $[P_n^+C^{n-}]_{org}$ value was obtained from the appropriate isotherm. The value of $[C^{n-}]_{aq}$ was calculated to include the contribution from the buffer. The constant K_1 was estimated from the chromatographic results obtained in the absence of pairing ion for each solute. Using an iterative computer program (HOOKE) to produce the best least-squares fit of the experimental data k' *vs.* $[P_n^+C^{n-}]_{org}$, the composite constant $K_2K_{IE}^{1/n}$ was evaluated for each solute and pairing ion. The corresponding graphs of k' *vs.* $[P_n^+C^{n-}]_{aq}$ using those derived constants are shown in Fig. 3 as full lines.

To demonstrate further the fit of the proposed model, Figs. 4(a–d) show experimental (dotted lines) and calculated (full lines) values of k' as a function of adsorbed pairing ion concentration $[P_n^+C^{n-}]_{org}$. The experimental curves in these plots indicate more forcefully that k' continues to decrease while $[P_n^+C^{n-}]_{org}$ is essentially constant.

The calculated lines in Figs. 3 and 4 show good agreement with the experimental values in terms of the general shape of the curve produced, especially in the region of decreasing k'. The position of the predicted maxima on the pairing-ion concentration scale do not consistently lie above or below the experimental values, indicating some error in the values allocated to the variables in eqn. 9. The calculated curve was found to be insensitive to the value of A_s but was highly sensitive to $[P_n^+C^{n-}]_{org}$ obtained from the isotherms. The experimental data points for the more hydrophobic naphthoic and 4-ethylbenzoic acids were not sufficiently representative of the parabolic shape of the curve to enable values of K_2K_{IE} to be obtained for the cetrimide pairing ion.

That the magnitudes of the experimental and calculated k' values are in agreement over such widely different values based on the best value of one adjustable constant is taken as evidence that the proposed ion-exchange–desolvation process is of major significance in the retention of the ionised acids in C_{18}–aqueous pairing-ion systems. The optimum values of the composite $K_2K_{IE}^{1/n}$ constant are shown in Table I, together with the average value of K_1 obtained for a particular solute in the absence of pairing ion. The K_2K_{IE} values represent the ion-exchange–desolvation process and are seen to increase with the hydrophobicity of solute for a given pairing ion, as does

TABLE I

K_1 AND $K_2K_{IE}^{1/n}$ VALUES CALCULATED TO PRODUCE THE BEST FIT OF EXPERIMENTAL DATA

Solute	$K_1 \cdot 10^8$	$K_2K_{IE}^{1/2}$ *(TMA)* $\cdot 10^5$	K_2K_{IE} *(TEA)*	$K_2K_{IE}^{1/2}$ *(TBA)* $\cdot 10^4$	K_2K_{IE} *(CTAB)*
BA	2.28	2.18	0.103	1.69	0.104
2,4 DMBA	9.52	11.48	0.519	5.76	0.208
NA	15.7	18.76	1.05	16.5	
4 EBA	24.4	26.23	1.36	17.9	

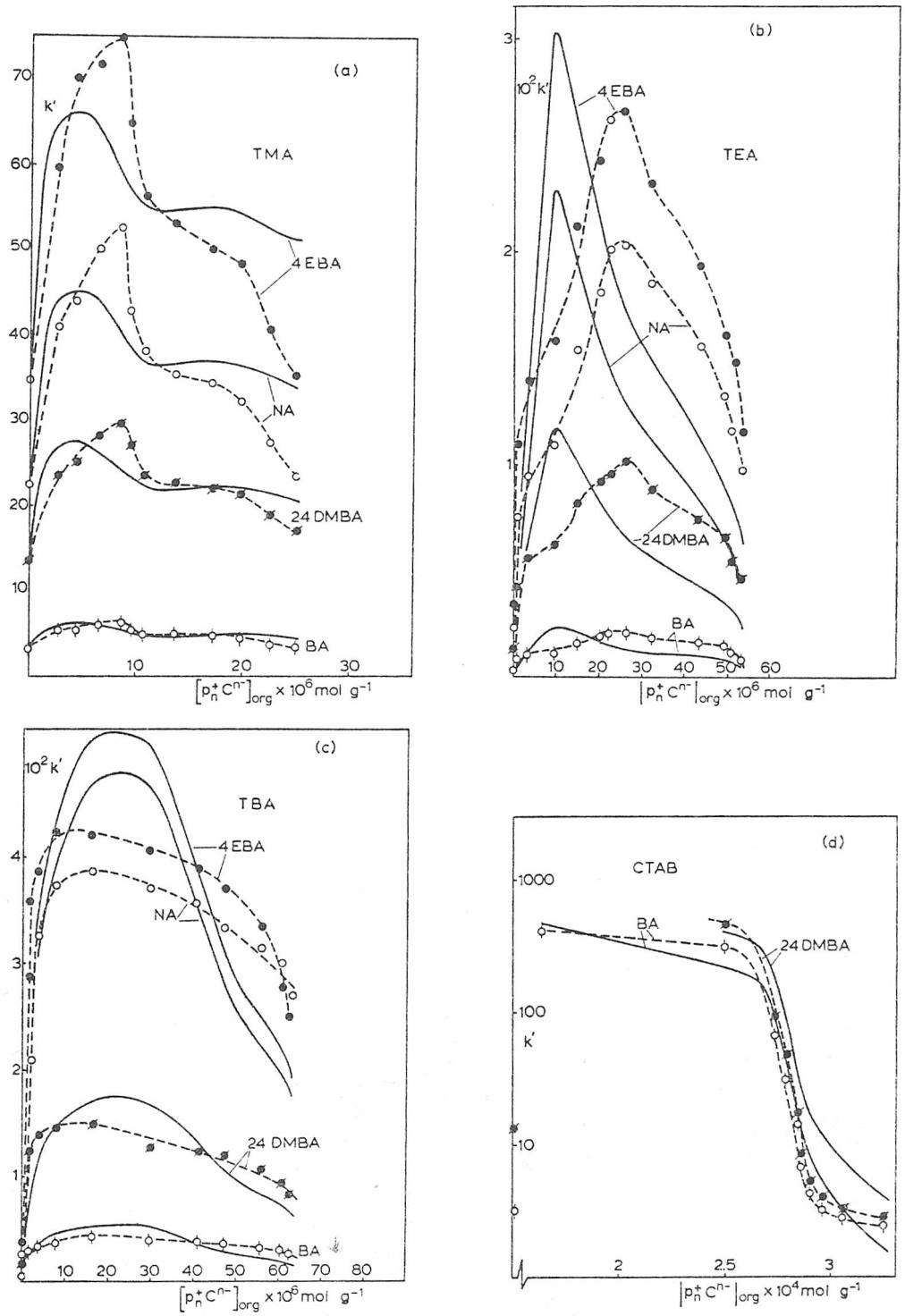

Fig. 4. Plots showing the variation of capacity factors (k') with adsorbed pairing-ion concentration for the four acidic solutes and various pairing ions (a–d). Dashed lines and full lines as in Fig. 3.

K_1. Considerable fluctuation, however, is apparent for the same solute as a function of pairing ion. There is no regular increase in $K_2K_{IE}^{1/n}$ with hydrophobicity of pairing ion as would be expected in an ion-pairing phenomenon where the K_2 representing desolvation would refer to the ion pair. In order to separate the components of desolvation and ion exchange, the approximation is made that $K_1 = K_2$, *i.e.* that the desolvation energy of a solute is the same under ion-exchange as under non-ion-exchange conditions. This enables the K_2 term, which in any case should be constant on this mechanism between pairing ions for any solute, to be eliminated and the K_{IE} constant to be evaluated taking into account the particular value of (n) for the pairing salt used. These results are shown in Table II. The K_{IE} values are substantially constant for all solutes within a given pairing ion, and even for different pairing ions the values are of comparable magnitude. This is taken to indicate that the electrostatic binding thouhgt to be operative is the same for all pairing ions and solutes, with the $K_2K_{IE}^{1/n}$ term dictating retention and resolution since in all cases this term is much larger than K_1. It must be stated that the $K_2K_{IE}^{1/n}$ values obtained for cetrimide, even for those solutes where an acceptable fit was obtained, are likely to be low owing to the lack of data at very high k' values.

TABLE II

K_{IE} VALUES CALCULATED FOR SOLUTE-PAIRING ION SYSTEMS ASSUMING $K_1 = K_2$

Solute	$K_{IE} \cdot 10^{-6}$			
	TMA	*TEA*	*TBA*	*CTAB*
BA	0.922	4.54	55.4	4.50
2,4 DMBA	1.45	5.45	36.3	2.60
NA	1.41	6.67	100.9	
4 EBA	1.15	5.58	53.8	

The behaviour of 5HMF in the presence of pairing ion yields further evidence of the surface coverage aspect of the proposed mechanism. In the absence of an ion-exchange mechanism, eqns. 9 and 10 reduce to

$$k' = \frac{K_1}{V_m}(A_s - [P_n^+ C^{n-}]_{org} A_P) \tag{11}$$

The plot of k' against $[P_n^+ C^{n-}]_{org} A_P$ of all pairing ions for 5HMF is shown in Fig. 5. While eqn. 11 predicts a constant gradient of K_1/V_m, the data points for three of the four pairing ions used lie on a curve of constantly decreasing slope. This may indicate a changing desolvation constant owing to a different alkyl surface being formed progressively owing to adsorption of pairing ion. The tetramethylammonium pairing ion appears not to conform to this pattern and no explanation can be given. The results of 5HMF indicate one possible oversimplification in the proposed mechanism, in that only the C_{18} surface is considered with regard to solute desolvation.

In the light of these results, the proposed ion-exchange–desolvation mechanism appears to account for the experimentally observed variation of k' with pairing-ion concentration. It is evident that both the concentration and type of pairing ion have

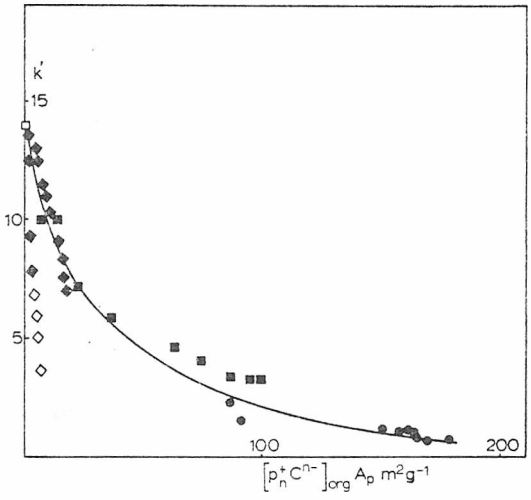

Fig. 5. Plot showing the variation of capacity factor for 5-hydroxymethylfurfuraldehyde as a function of the area of C_{18} surface calculated as being covered by various pairing ions at the concentrations studied. ◇, TMA; ◆, TEA; ■, TBA; ●, CTAB.

a marked effect and that prediction of retention is difficult without detailed knowledge of the isotherm characteristics and the operative ion-exchange constants. There appears no difference in the mode of action of the various pairing ions other than in their adsorption characteristics. It is likely that the addition of an organic modifier to bring the k' values to reasonable magnitudes will alter the isotherm shape[27] as well as reducing the very powerful hydrophobic effect exhibited in the present work. Further investigation is required to verify this quantitatively using the proposed mechanism and derived equation, especially in the case of very highly adsorbed pairing ions such as cetrimide. It is possible that the change in isotherm with organic modifier concentration will enable rationalisation of the very variable chromatographic ion-pairing results obtained by different investigators.

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ION-EXCHANGE–DESOLVATION MECHANISM ON OCTADECYL SILICA USING ANIONIC HYDROPHOBIC PAIRING IONS

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SUMMARY

The adsorption isotherms for several anionic hydrophobic pairing ions in water on ODS Hypersil are reported. The previously proposed retention model involving ion exchange and desolvation is assessed for various ionised basic solutes by comparing experimental capacity factors with those calculated on the basis of the proposed model as a function of pairing-ion concentration. Relative ion-exchange constants are calculated and shown to be constant for all pairing ions and solutes. Addition of acetonitrile as organic modifier in aqueous solution is shown to alter both the loading and the isotherm shape for both sodium laurylsulphate and cetrimide. Using appropriate solutes, it is shown that the ion-exchange–desolvation model applies quantitatively to the mixed solvents studied.

INTRODUCTION

Although successful chromatographic separations of ionogenic species have been achieved since the inception of paired-ion liquid chromatography, the retention and separation mechanisms involved remain unclear. The variety of nomenclature applied to the technique[1] is indicative of this uncertainty.

Previous attempts to explain the mechanism of paired-ion chromatography using hydrophobic pairing ions have been on the basis of ion pairs formed on the C_{18} surface[2,3] or in the eluent[4,5]. The equation relating eluent pairing-ion concentration to experimentally observed capacity factors do not predict the parabolic behaviour experimentally observed by various workers[2,6,7]. Neither do these approaches consider the adsorption of pairing ion on to the C_{18} surface other than in competition with the solute species[2,3]. Increasing evidence that the pairing ion is adsorbed by the stationary phase to a considerable degree has supported an alternative retention mechanism involving ion exchange between adsorbed ionised pairing ions and ionised solutes[8–11]. Quantitative treatment of this model has been restricted to relating capacity factors to measured adsorbed-pairing-ion concentrations over very limited concentration ranges and for unique pairing ions[8,10,11]. Also, in this ion-exchange model no consideration is given to the hydrophobicity of the solute determining

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desolvation on the C_{18} surface. Other mechanisms describing the retention of ionogenic solutes have not been treated quantitatively[1].

In a recent communication a retention model incorporating ion exchange and desolvation was proposed[1,2] and the dependence of capacity factor on adsorbed-pairing-ion concentration was derived. Experimental data involving several different cationic pairing ions and acidic solutes were found to be in broad agreement with the proposed model over a very wide range of pairing-ion concentrations, and the parabolic nature of the dependence of capacity factor on pairing-ion concentration was demonstrated.

The present investigation extends this work in two ways. It verifies that anionic pairing ions, which are perhaps more generally used for chromatography of bases, show similar adsorption isotherms to those obtained for cationic species, and that the ion-exchange–desolvation model also accounts for the chromatographic behaviour of basic solutes on such phases. It also examines the effect of organic modifier on adsorption isotherms with subsequent chromatography. In the present work a series of variously substituted amines have been used as solutes in order to verify the general application of the model and treatment.

THEORY

The ion-exchange–desolvation model may be represented as follows. Pairing ion is adsorbed from an eluent to an extent governed by its hydrophobicity and in some cases largely independently of its eluent concentration. Solutes, oppositely charged with respect to the pairing ion, interact electrostatically with the adsorbed pairing ion. Retention of the solute by the C_{18} stationary phase is largely as a result of desolvation consequent on the neutralisation of coulombic charges. Occupation of the C_{18} surface by adsorbed pairing ions modifies the surface area available for desolvation of the solute.

This model embodies previously suggested ion-exchange ideas and also includes an attempt to allow for the conventional desolvation processes normally thought to be operative in reversed-phase liquid chromatography. An equation of the form:

$$k' = \frac{1}{V_m}(A_s - [P_n^+ C^{n-}]_{org} A_p)\left\{K_1 + K_2\left(\frac{K_{IE}\,[P_n^+ C^{n-}]_{org}}{[C^{n-}]_{aq}}\right)^{1/n}\right\} \quad (1)$$

has been derived on the basis of this model for the case of cationic pairing ions.

The parameter k' is the capacity factor for a given solute and V_m the void volume of the column. The factor A_p is the area occupied by the adsorbed pairing ion per mole. K_1 and K_2 are the desolvation constants of the solute in absence and presence of pairing ion, respectively, and K_{IE} is the ion-exchange equilibrium constant. $P^{\pm}$ represents a univalent pairing ion of either charge and $C^{n\pm}$ a possibly multivalent counter-ion. A_s represents the area of the stationary phase available for desolvation in the absence of pairing ion. In the case of a monovalent counter ion, eqn. 1 may be simplified and expanded to

$$k' = \frac{1}{V_m}(A_s K_1 - K_1 [P^{\pm} C^{\mp}]_{org} A_p + \frac{K_2 K_{IE} A_s [P^{\pm} C^{\mp}]_{org}}{[C^{\mp}]_{aq}} - \frac{K_2 K_{IE} A_p [P^{\pm} C^{\mp}]_{org}^2}{[C^{\mp}]_{aq}} \quad (2)$$

Agreement between the parabolic behaviour of acid solutes using cationic pairing ions predicted by an equation of this form and experiment has already been established[12]. In the present work it is intended to show that the agreement extends to widely differing basic solutes with different anionic pairing ions.

EXPERIMENTAL

Apparatus and materials

The apparatus used has been described previously[12]. Columns for isotherm measurements were 250 × 5 mm I.D. and those used for the determination of capacity factors 50 × 5 mm I.D.

1-Pentanesulphonic acid (PANS) and 1-octanesulphonic acid (OCTS) were obtained as their sodium salts from Eastman-Kodak (Rochester, NY, U.S.A.). Sodium laurylsulphate (SLS), aniline hydrochloride (AN) and benzylamine (BZ) were obtained from BDH (Poole, Great Britain). Dopamine hydrochloride (DOP) was obtained from Sigma (St. Louis, MO, U.S.A.) and *dl*-10-camphorsulphonic acid (CAMS) as the sodium salt and L-adrenaline-D-hydrogentartrate (AD) from Koch-Light Labs. (Colnbrook, Great Britain). The stationary phase ODS-Hypersil, cetrimide (CTAB) and the various carboxylic acids, benzoic acid (BA), 2,4-dimethylbenzoic acid (2,4-DMA), 4-ethylbenzoic acid (4-EBA) and naphthoic acid (NA) together with the test neutral compound 4-hydroxymethyl furfuraldehyde (5HMF) were obtained as described previously. Acetonitrile was obtained from Rathburn (Walkerburn, Great Britain). Water was double distilled and all other reagents were of AnalaR or comparable quality.

Procedures

Adsorption isotherms for the four anionic pairing ions PANS, OCTS, CAMS and SLS on ODS-Hypersil were determined as before by adsorbing from a fixed concentration of pairing in aqueous solution, desorbing using ethanol and weighing the total amount recovered. This method was adopted after practically assessing other techniques of isotherm determination[3,9,13]. A similar procedure was adopted for isotherms measured in the presence of organic modifier.

Chromatographic measurements were carried out in a standard phosphate buffer, pH 1.5 for the cationic solutes and pH 7.4 for the anionic solutes measured on CTAB in mixed solvents. A flow-rate of 3 $cm^3\ min^{-1}$ was employed throughout, and the wavelength of measurement of all solutes was at 254 nm except when CAMS was used as pairing ion. In this case 235 mm was chosen as the wavelength of minimal background absorbance.

RESULTS AND DISCUSSION

Isotherm results

Adsorption isotherms for the four anionic pairing ions used in pure water are shown in Fig. 1 over an aqueous concentration range of 2–400 mol m^{-3}. With the exception of SLS, the isotherms are L type according to the Giles classification[14] and indicate a smooth rise to a plateau level characteristic of the particular pairing ion. At low concentrations these isotherms approach linearity. SLS, on the other hand, is

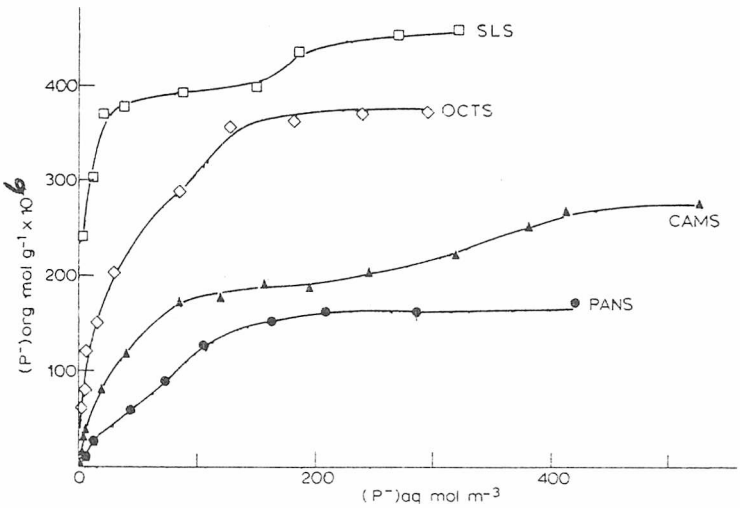


Fig. 1. Adsorption isotherms for various anionic pairing ions for the ODS Hypersil–water system. ●, PANS; ▲, CAMS; ◇, OCTS; □, SLS.

markedly H type, *i.e.* even at low aqueous concentrations very large concentrations of pairing ion are adsorbed at equilibrium. These results confirm the findings of our previous investigation in that the loading capacity of different pairing ions on ODS-Hypersil appears to be related to the hydrophobicity of the particular pairing ion. In this case, however, no attempt is made to correlate the loading capacity with any hydrophobic parameter in view of the difficulty of estimating the hydrophobicity of CAMS. The H-type behaviour of SLS observed after long equilibration times is analogous to that of the correspondingly surface-active cationic species (CTAB). Since most chromatographic separations involve the use of an organic modifier, it was thought useful to examine the effect of acetonitrile concentration on the adsorption properties of the two most highly adsorbed pairing ions used in this and the previous study, namely SLS and CTAB. The results are shown in Figs. 2a and b, respectively, for three concentrations of acetonitrile (0, 30 and 60 %, v/v). For both pairing ions the isotherm is markedly altered by the addition of acetonitrile. Firstly, the column loading is decreased, an effect which has been indicated by previous workers[1,11]. In addition, the shape of the isotherm is seen to be drastically altered. The H-type isotherms observed in aqueous eluent are L type in 30 % and approximate to linearity in the highest acetonitrile concentration used.

These results demonstrate the inadequacy of arbitrarily fitting adsorption data to particular isotherms. They further support the present approach of utilising directly determined adsorbed-pairing-ion concentrations when attempting to quantity chromatographic behaviour.

Chromatographic results

The variation of the capacity factors (k') of the various ionised solutes with pairing-ion concentrations both in eluent and adsorbed on to the organic stationary phase are shown in Figs 3a–h and 4a–h, respectively. Included in these figures are the results obtained for mixed eluents containing different proportions of organic mod-

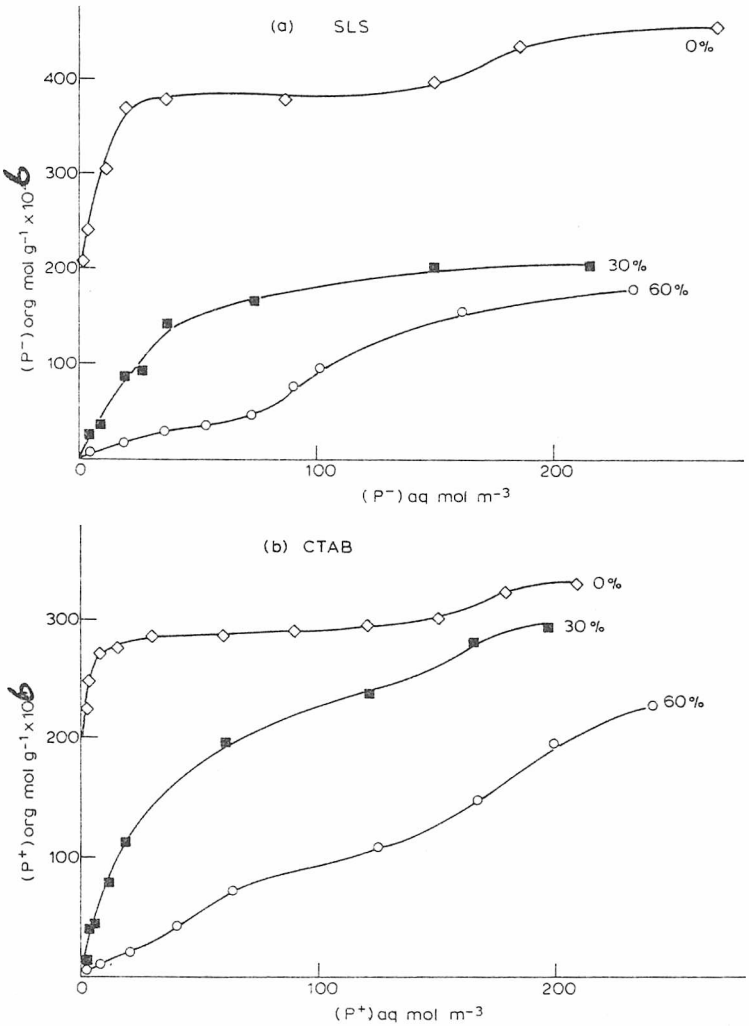


Fig. 2. Adsorption isotherms for (a) sodium laurylsulphate and (b) cetyltrimethylammonium bromide, for different concentrations of acetonitrile–water mixtures. ◇, Pure water; ■, 30% (v/v) acetonitrile; ○, 60% (v/v) acetonitrile.

ifier. Broken lines refer to the experimentally observed k' values and the solid lines represent the k' values calculated on the basis of the derived model.

As in the previous study of cationic pairing ions[12], K_1 values were determined from measurements of retention times in the absence of pairing ion. $[P^-_n\ C^{n+}]_{org}$ was obtained directly from the appropriate isotherm, and $[C^{n+}]_{aq}$ was estimated from aqueous pairing-ion concentration and buffer composition. The values of A_p were calculated as before from the dimensions of the individual solutes[15], and A_s was taken[16] as 142 $m^2\ cm^{-3}$. The V_m value for the column was estimated using sodium nitrate as an unretained solute[17]. Since the K_2K_{IE} term could not be evaluated analytically, an iterative curve-fitting program (HOOKE) was employed to select the value of this composite constant which would yield the best least-squares fit of the equation with the experimental data.

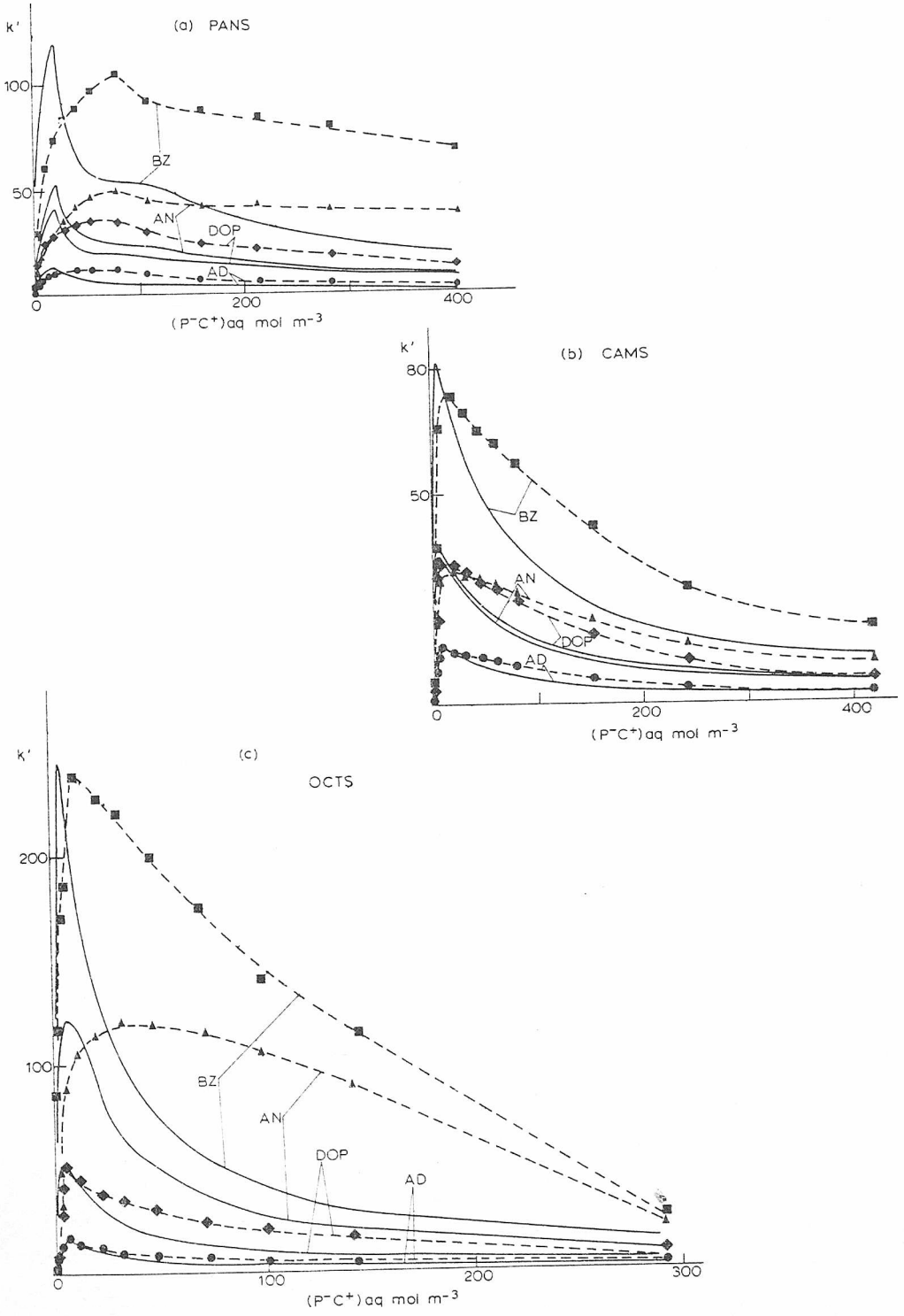
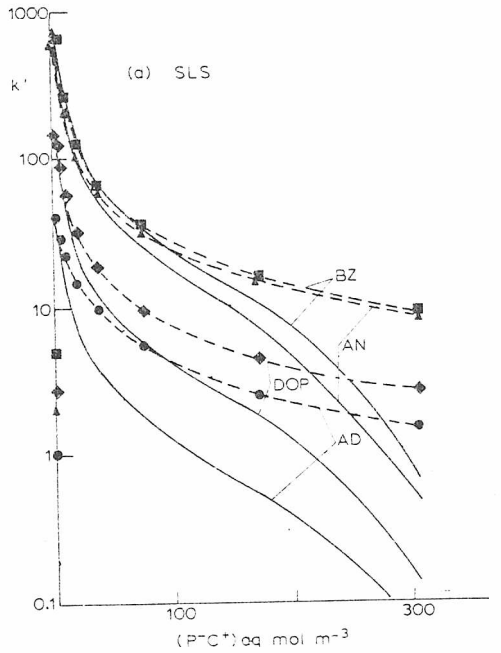

k'
(a) PANS
100
50
BZ
AN
DOP
AD
0
200
400
(P⁻C⁺)aq mol m⁻³
k'
(b) CAMS
80
50
BZ
AN
DOP
AD
0
200
400
(P⁻C⁺)aq mol m⁻³
k'
(c)
OCTS
200
100
BZ
AN
DOP
AD
0
100
200
300
(P⁻C⁺)aq mol m⁻³

Fig. 3.



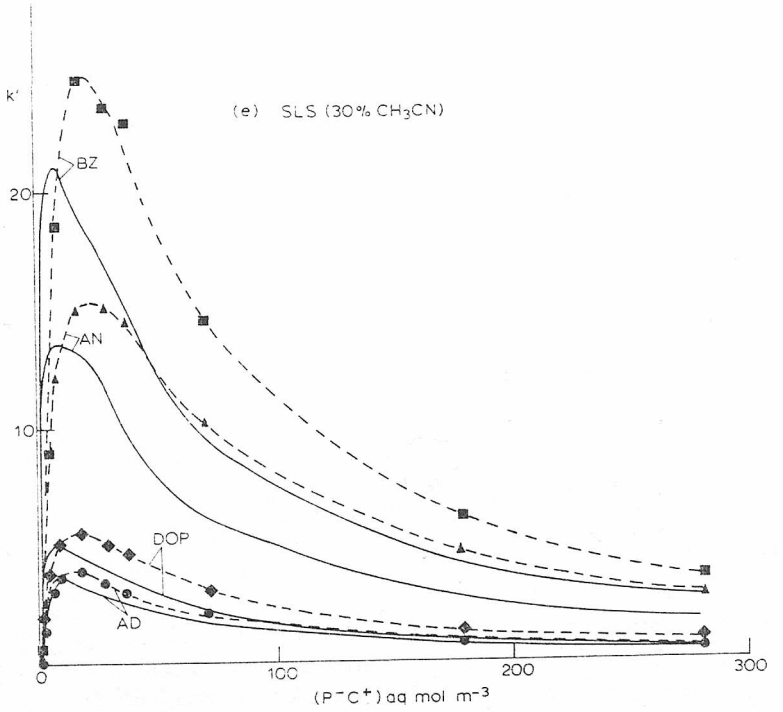


Fig. 3.

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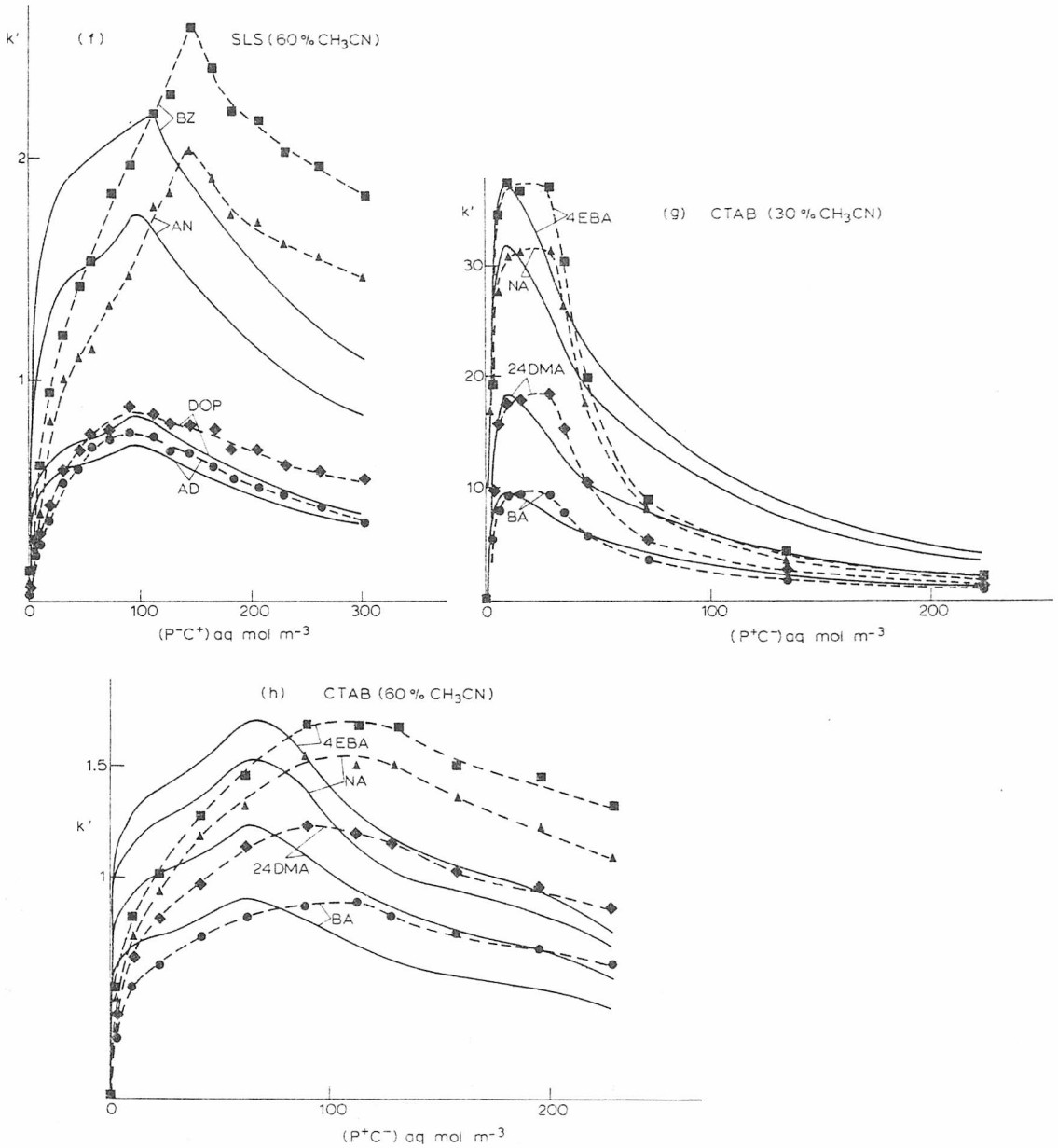


Fig. 3. Plots showing the variation of capacity factor (k') with aqueous pairing-ion concentration for the different solutes and pairing ions used. Broken lines represent experimental results. Solid lines represent calculated curves.

The experimental results clearly indicate a parabolic dependence of k' on pairing-ion concentration both in the mobile and stationary phases, and generally good agreement between the shapes of experimental and calculated curves is obtained. In the case of the surfactant pairing ion, SLS, only the decreasing part of the curve can be observed experimentally owing to the high adsorption at all concen-

trations exhibited for this pairing ion on the C_{18} surface, confirming the results previously obtained for the surfactant pairing ion, CTAB.

The increase of k' with aqueous pairing-ion concentration is interpreted as due to an increasing adsorption of pairing ion by the C_{18} surface, and the correlation of the magnitudes of k' for a given solute with different pairing ions supports the ion-exchange aspect of the present model. The decreasing section of the curve is accounted for in the mathematical treatment of the model by the sum of two effects, namely decreased C_{18} surface available for desolvation and increasing eluent counter-

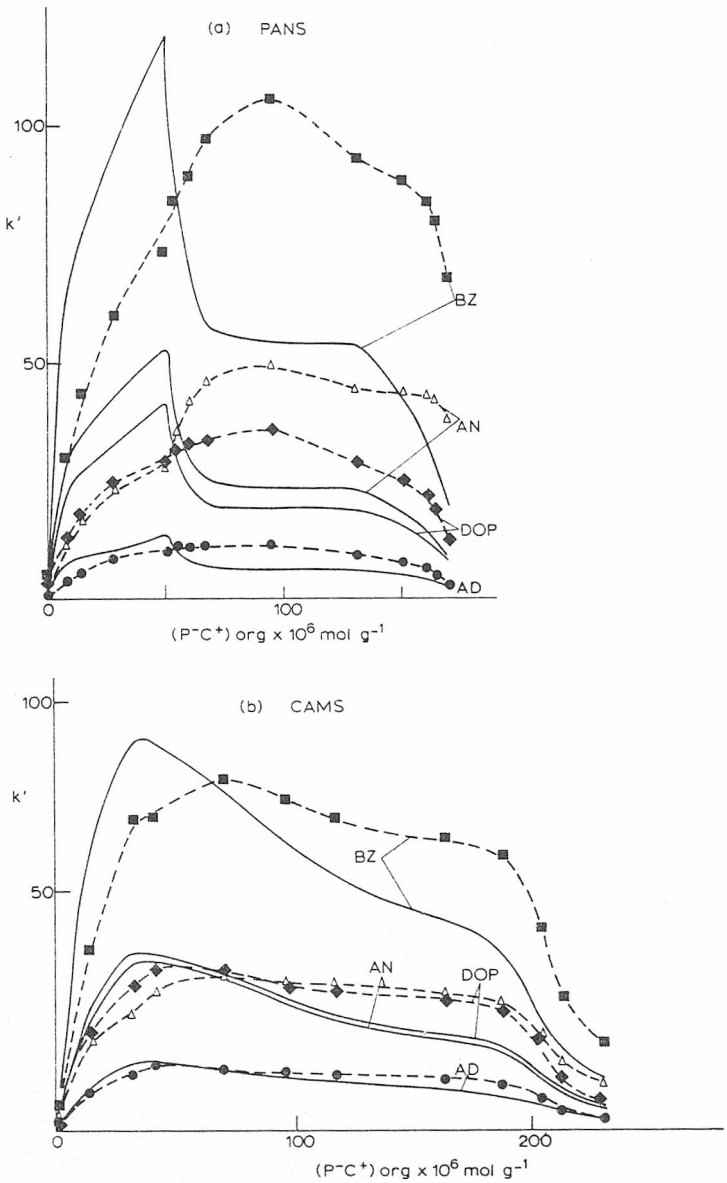
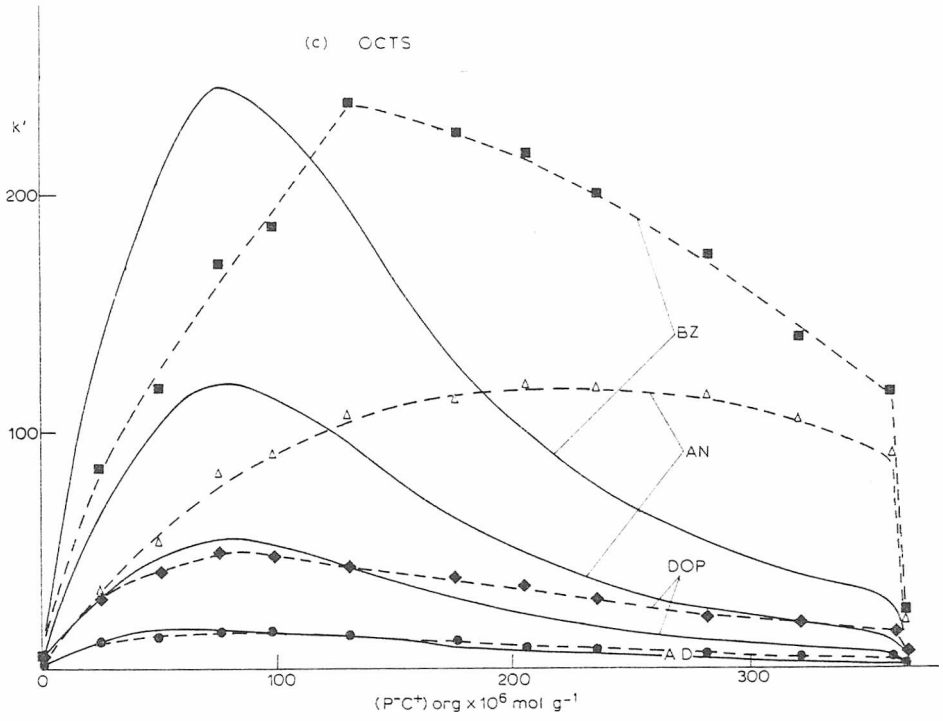


Fig. 4.

(Continued on p. 184)


(c) OCTS
k'
200
100
0
100
200
300
BZ
AN
DOP
AD
(P⁻C⁺) org x 10⁶ mol g⁻¹

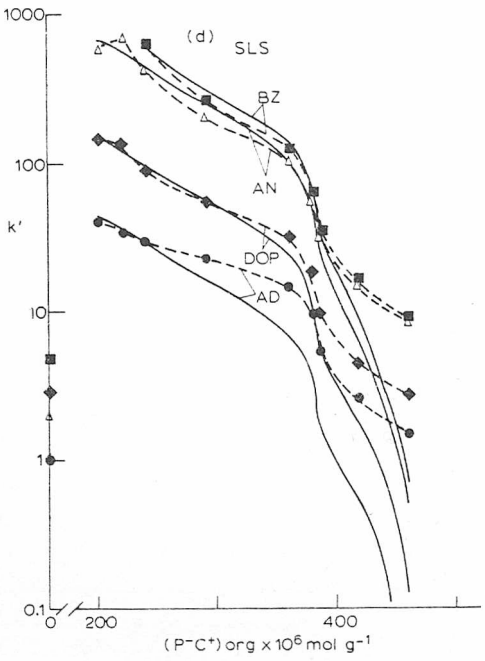
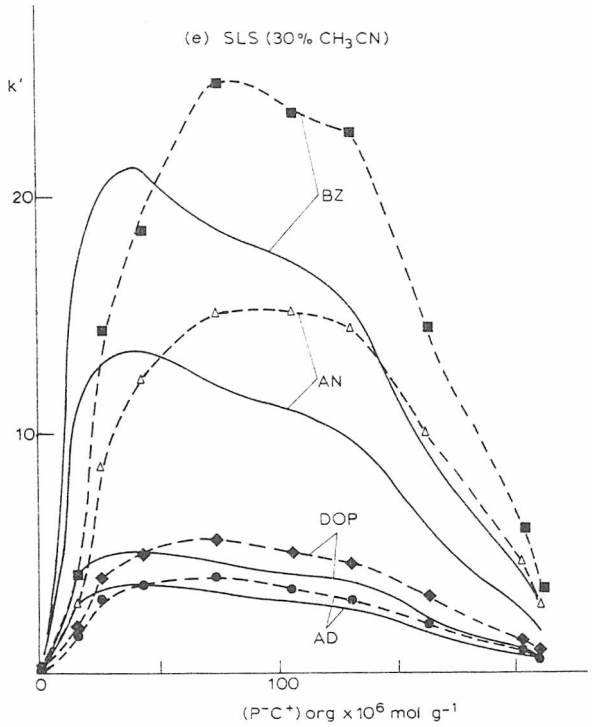

(d) SLS
1000
100
10
1
0.1
k'
0
200
400
BZ
AN
DOP
AD
(P⁻C⁺) org x 10⁶ mol g⁻¹

Fig. 4.



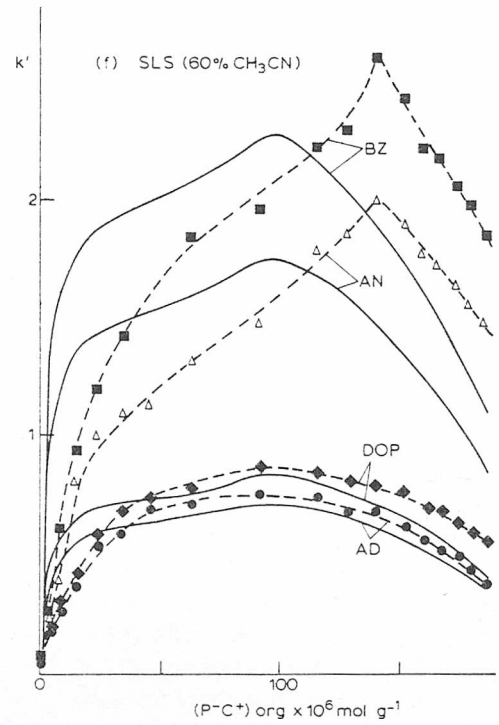


Fig. 4.

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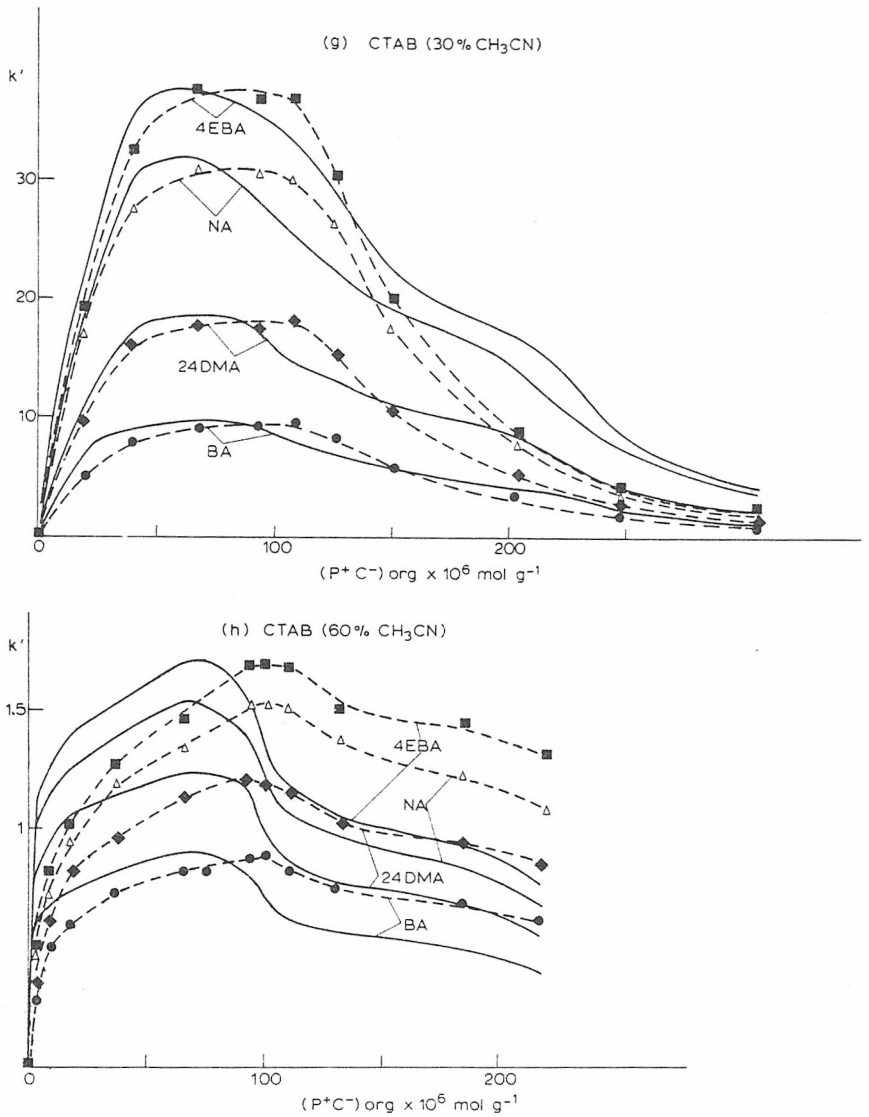

Fig. 4. Plots showing the variation of capacity factor (k') with organic-pairing-ion concentration for the different solutes used. Broken lines represent experimental results. Solid lines represent calculated curves.

ion concentration as a result of added pairing-ion salt. This latter effect is demonstrated in the case of SLS where the organic concentration of pairing ion is essentially constant, as evidenced by the plateau region of the isotherm. The effect of counter-ion in this model is similar to that of the original ion-exchange equilibrium model[11] but is more realistic in that the addition of pairing-ion salt to the eluent will firstly alter the C_{18} surface area of the phase ratio $(A_s - [P_n^{\pm} C^{n\mp}]\, A_p)/V_m$ and, if this is not possible owing to equilibrium loading of the surface having been achieved, act as a conventional counter-ion.

In our previous treatment of various carboxylic acids, the assumption was

made that $K_1 = K_2$ which enabled the values of K_{IE} to be uniquely estimated. While this may be approximately true in the case of solutes which have appreciable lipophilicity in the ionised state, it will be less true in the case where the solute retention in the absence of pairing ion is very small. In this more general case, however, the assumption may be made that K_2/K_1 is constant for a given pairing ion, which allows relative values of K_{IE} to be estimated, *i.e.* K'_{IE}. This constant will still provide information on the coulombic interactions of solute and adsorbed pairing ion. This assumption appears to be justified in recent studies of the retention of carboxylic acids as a function of pH where the k' values of solutes have been measured in both non-ionised and fully ionised states[10,18]. Values of $K_2 K_{IE}$ and K_1 are shown in Table I and the calculated values of K'_{IE} involving the above assumption are shown in Table II. It can be seen from the latter results that the K'_{IE} values are remarkably constant, not only for a given pairing ion but also among different pairing ions. This lends support to the concept of a constant charge neutralising effect preceding a particular desolvation tendency which is characteristic of a given solute.

TABLE I
K_1 AND $K_2 K_{IE}$ VALUES CALCULATED TO PRODUCE THE BEST FIT OF EXPERIMENTAL DATA

Solute	$K_1 \times 10^8$	$K_2 K_{IE} \times 10^2$			
		PANS	*CAMS*	*OCTS*	*SLS*
AD	0.711	3.757	2.827	1.194	1.293
AN	1.41	15.12	7.295	8.718	20.153
DOP	1.99	11.49	7.484	3.825	4.405
BZ	3.52	34.093	16.656	17.429	27.35

TABLE II
K'_{IE} VALUES CALCULATED FOR SOLUTE-PAIRING SYSTEMS ASSUMING K_2/K_1 IS CONSTANT

Solute	$K'_{IE} \times 10^{-6}$			
	PANS	*CAMS*	*OCTS*	*SLS*
AD	5.284	3.976	1.679	1.819
AN	10.723	5.174	6.183	14.56
DOP	5.774	3.761	1.922	2.214
BZ	9.685	4.732	4.732	7.77

In the case of eluents incorporating acetonitrile, the values of k' in the absence of pairing ion are very small and for the higher concentrations of acetonitrile apparently negative[19]. For the mixed solvent measurements, the $K_2 K_{IE}$ values are shown as a function of acetonitrile concentration in Table III. As would be expected, their values are lower than in the pure aqueous solvent owing to adsorption of acetonitrile in addition to pairing ion by the surface.

TABLE III
$K_2 K_{IE}$ VALUES CALCULATED TO PRODUCE THE BEST FIT OF EXPERIMENTAL DATA IN MIXED ORGANIC/AQUEOUS SOLVENT

Solute	$K_2 K_{IE} \times 10^2$ *(SLS)*		*Solute*	$K_2 K_{IE} \times 10^2$ *(CTAB)*	
	30% (v/v) Acetonitrile	*60% (v/v) Acetonitrile*		*30% (v/v) Acetonitrile*	*60% (v/v) Acetonitrile*
AD	0.772	0.614	BA	1.228	0.748
AN	2.932	1.521	2,4-DMA	2.376	1.026
DOP	1.087	0.702	NA	4.139	1.270
BZ	4.582	1.947	4-EBA	4.876	1.415

As in our previous study, the behaviour of a single uncharged compound 5HMF agrees only qualitatively with the derived equation. In the absence of a K_{IE} term, eqn. 2 may be written:

$$k' = \frac{K_1}{V_m} (A_s - [P_n^{\pm} C^{n\mp}]_{org} A_p) \tag{3}$$

The combined results of the behaviour of 5HMF on Hypersil ODS in the presence of an equilibrium concentration of adsorbed cationic and anionic pairing ion in aqueous solvents is shown in Fig. 5 as the variation of k' with $[P_n^{\pm} C^{n\mp}]_{org} A_p$.

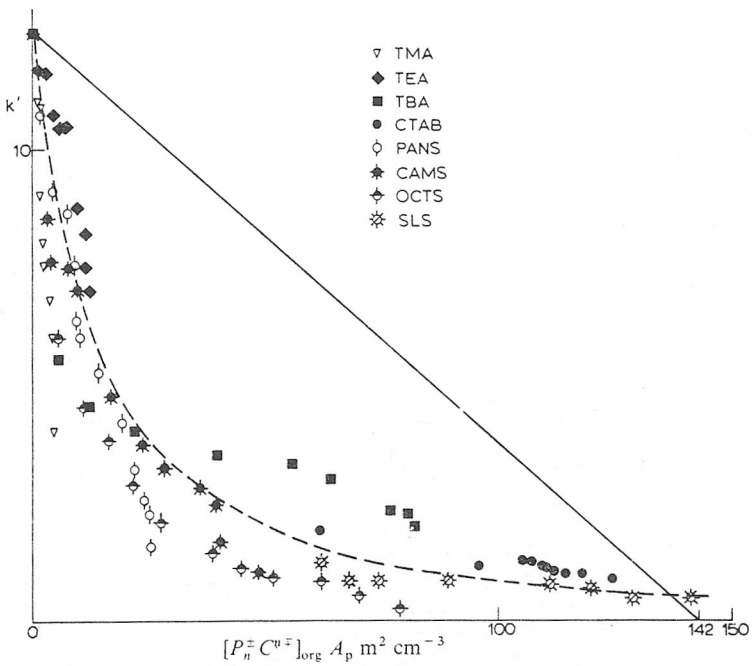

Fig. 5. Plot showing the variation of capacity factor for 5HMF as a function of the area of the C_{18} calculated as being covered by various pairing ions at the concentrations studied. Broken line represents experimental results. Solid line represents eqn. 3.

The theoretical line of this equation appears to underestimate the surface exclusion effect of the adsorbed pairing ions on a neutral species. For all pairing ions at all concentrations, there is a marked reduction of the 5HMF capacity factor, which tends to a small constant value. It is possible that the surface area aspect of the present model is quantitatively inadequate owing to our incomplete understanding of the physical nature of the C_{18} surface in the presence and absence of organic modifiers and the pairing ions[20,21]. This limitation does not invalidate the generality of the proposed model and any alteration in the A_s and A_p values used would only have the effect of altering the absolute magnitudes of the derived constants.

CONCLUSIONS

The present results for anionic pairing ions of different hydrophobicities confirm the findings of our previous study for cationic species, namely that the loading of the C_{18} phase with pairing ion in aqueous eluents is complex. This is especially true in the case of long-chain pairing ions having surfactant properties. These show high adsorption at very low eluent pairing-ion concentrations. The use of such pairing ions may produce unacceptably high k' values when used with solutes which have appreciable retention in the absence of pairing ion, unless appreciable concentrations of organic modifiers are used to change the H-type behaviour. Less strongly adsorbed pairing ions provide a more sensitive method of adjusting retention times by pairing-ion concentration.

The ion-exchange–desolvation model appears adequately to represent the behaviour of both anionic and cationic solutes in the presence of pairing ion. The magnitudes of the constants relating to the ion-exchange aspect of the proposed mechanism indicate that the electrostatic binding is approximately constant for all solutes and pairing ions. The model also predicts the chromatographic behaviour adequately in mixed eluents containing both water and organic modifier.

The behaviour of 5HMF as a fairly polar neutral solute shows a marked decrease in retention with increasing pairing-ion concentration for all pairing ions studied, which can be of use in the separation of charged and uncharged solute species by the ion-pairing technique[22]. The quantitative predictions of the model described do not adequately predict the behaviour of our neutral test substance and this, it is believed, is due to the difficulty of quantifying the phase ratio in the presence of pairing ion.

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AN ION PAIRING H.P.L.C. INVESTIGATION OF THE ACIDITY PRODUCED IN THERMALLY DEGRADED DEXTROSE SOLUTION

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The literature contains conflicting evidence for the source and nature of the acidic product(s) responsible for the pH decrease generally observed during the heating of dextrose solutions. It is widely believed that the acid is formed by subsequent degradation of 5-hydroxymethylfurfuraldehyde (5HMF) (Tahir and Cates 1974; Sturgeon et al 1980). It has also been suggested that the acidity is produced by a second reaction product unrelated to 5HMF (Taylor and Sood 1978). Dextrose solutions (10% w/v) were heated at 120°C in sealed ampoules for times up to 6 hours. At intervals, ampoules were removed, cooled and the contents subjected to reverse phase chromatography. A capped ODS stationary phase ODS Hypersil was used with water as solvent and detection was at 254 nm. On the basis of the time dependence of peak heights produced, two major intermediates (I_1, I_2) and two acidic products (A_1, A_2) were detected in addition to the main 5HMF product. A_1 and A_2 were designated acidic products due to their elution at or before the solvent front coupled with the observed increase in their retention times when various quaternary ammonium pairing ions were added to the chromatographic solvent. The addition of pairing ion at different concentrations to the chromatographic solvent markedly improved the retention and separation of A_1 and A_2. The behaviour of A_1 and A_2 followed that shown to be general for carboxylic acids in presence of pairing ion (Hung and Taylor 1980). The use of different pairing ions also allowed a more rigorous comparison of the behaviour of A_1 and A_2 with that of previously suggested acid species namely acetylacrylic, levulinic and formic acids. The observed behaviour did not correspond with any of these. No standard metasaccharinic acid was available but A_1 and A_2 both showed appreciable maximal absorption at 250 nm which was taken to be indicative of a ketocarboxylic acid.

Chromatographic behaviour of A_1 and A_2 without pairing ion but in the presence of solvents of different pH values showed much greater retention at low pH than any of the suggested species and indicated a pKa value for both A_1 and A_2 of approximately 3.0. Isolation of peaks I_1 and I_2 followed by heating in solution and subsequent chromatography with an ion pairing solvent produced peaks corresponding to A_1, A_2 and 5HMF, confirming the intermediate nature of I_1 and I_2. Heating aqueous solutions of 5HMF (0.027% w/v) for 8 hours at 120°C in sealed ampoules with subsequent chromatography in ion pairing solvents showed no decomposition of that product, no evidence of A_1 or A_2, nor did the pH of the solution change. The pH decrease generally observed in dextrose was observed to be kinetically consistent with the rise in concentration of the acid products A_1 and A_2. These observations appear to support the contention that the acidity in heated Dextrose solutions is due to acid products formed directly from the intermediates. It has recently been observed, however, following prolonged storage of degraded Dextrose solutions in presence of air at room temperature, that 5HMF originally present appears to be converted to acidic compounds. The complexity of the reaction is further demonstrated by the observation that on heating the isolated intermediates I_1 and I_2, the solution assumes a brown colouration which supports the suggestion (Fleming et al 1969) that an α-diketohexose intermediate itself polymerises and explains the observation that autoclaved dextrose solutions may exhibit brown colouration while containing very small concentrations of 5HMF.

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A CHROMATOGRAPHIC QUALITY CONTROL PROCEDURE BASED ON HPLC FOR 5-HYDROXYMETHYLFURFURAL IN AUTOCLAVED D-GLUCOSE INFUSION FLUIDS

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SUMMARY

A specific quantitative chromatographic assay method is described for 5-hydroxymethylfurfuraldehyde (5HMF) in degraded D-glucose solutions incorporating an internal standard. Comparison is made between the chromatographic method and the official direct ultraviolet absorption measurements at 284 nm for solutions of D-glucose degraded to different extents. Hospital samples of autoclaved Dextrose Injection, 5% w/v, are also examined by both methods and it is demonstrated that quite different results can be obtained depending upon the conditions of measurement. It is demonstrated that the direct absorption method does not provide an adequate limit test for compounds related to 5HMF.

INTRODUCTION

D-glucose solutions for use as intravenous infusion fluids are liable to decomposition during and after autoclaving (1, 2). Extensive investigations have been carried out on the kinetics of the degradation (3) and the reaction mechanism has been shown to be complex by both spectrophotometric (4) and chromatographic techniques (5).

It is well established that a main decomposition product during the D-glucose decomposition is 5-hydroxymethylfurfuraldehyde (5HMF) and it is on this fact that the pharmacopoeial limit test for D-glucose infusion fluid is based. This limit test involving direct measurement of the absorbance of the solution at 284 nm after suitable dilution purports to be a measure of 5HMF and related compounds. It is also known (2) that D-glucose solutions on autoclaving become acid and, though several suggestions with supporting evidence have been made as to the nature of the acid, no unequivocal identification has yet been made of this species (5, 6). Its concentration, however, is limited in the British Pharmacopoeia by a pH limit and in the European Pharmacopoeia by a simple titration method.

It would appear that the quality control of such a widely used infusion fluid is based on an extremely non-specific, albeit simple set of tests and it is the purpose of the present work to present a comparison between the official limit test for 5HMF and its related products and a

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chromatographic method depending on a straightforward high performance liquid chromatographic (hplc) method. In view of the extremely wide pharmaceutical use of this technique, it is likely that it will become increasingly available to the quality control pharmacist. It is further intended in the present work to indicate shortcomings in the present official method and to provide evidence that a more rigorous quality control test could be readily developed which would deal with the general impurity problem in autoclaved D-glucose solutions more completely than the present very superficial quality control applied.

EXPERIMENTAL

Chromatographic measurements were made using a Waters Associates M6000A pump and Model 440 fixed wavelength (254 nm) ultraviolet detector. Measurements at wavelengths other than 254 nm were recorded using a Pye Unicam LC-3 ultraviolet absorbance detector. Injection was by a Rheodyne 7125 valve fitted with a 20 μl loop. Various column lengths were used ranging from 70 to 200 mm depending upon the resolution and retention times required and columns were 4·6 mm internal diameter. Columns were slurry-packed with ODS Hypersil (Shandon) as required and typically gave a plate efficiency in excess of 5000 per 100 mm of column length. Solvents used were distilled water with and without disodium hydrogen phosphate as buffer and acetonitrile (Rathburn Chemicals). Tetrabutylammonium sulphate for use as pairing ion was obtained from Aldrich Chemical Co. Ultraviolet spectra were recorded by the stopped flow technique using a 10 μl flow cell and a Pye Unicam SP-1800 spectrophotometer. D-glucose solutions were degraded by sealing 5% solutions in glass ampoules and heating in an air oven for various periods of time. Samples of D-glucose solutions having been autoclaved by an accepted regimen were obtained from Aberdeen Royal Infirmary.

RESULTS AND DISCUSSION

The complexity of the degradation pattern of D-glucose is demonstrated in Fig. 1, where chromatograms of D-glucose solution decomposed for 1 h at 120°C are shown with detection at several wavelengths ranging from 220 nm to 300 nm. While the relative magnitudes of the various peaks will alter with extent of reaction as has been previously reported (5), Fig. 1 demonstrates the following:

(a) Several ultraviolet absorbing species are present in such solutions besides 5HMF. Peaks labelled 3, 4 and 5 are intermediates in the decomposition reaction and the single peak at the solvent front of the chromatogram is identified as the unretained anion of an acidic product of the reaction (5).

(b) The sensitivity of the detection system depends markedly on wavelength of measurement and shows the limitations of a single wavelength measurement as an estimation of impurity levels in such solutions. For example, the measurement of absorbance at 284 nm will measure 5HMF with maximum sensitivity, but will also measure intermediate 4 while intermediates 3 and 5 will not be observed.

These results indicate the potential inaccuracies that may be encountered in using the pharmacopoeial limit test in the quality control of autoclaved D-glucose solutions in that not all related compounds will be measured. It is, however, also the case that in the literature several workers have utilized the absorbance of such solutions at 284 nm as being a measure of the 5HMF concentration. This may be equally inaccurate since it is apparent from Fig. 1 that certain of the intermediates do absorb at 284 nm.

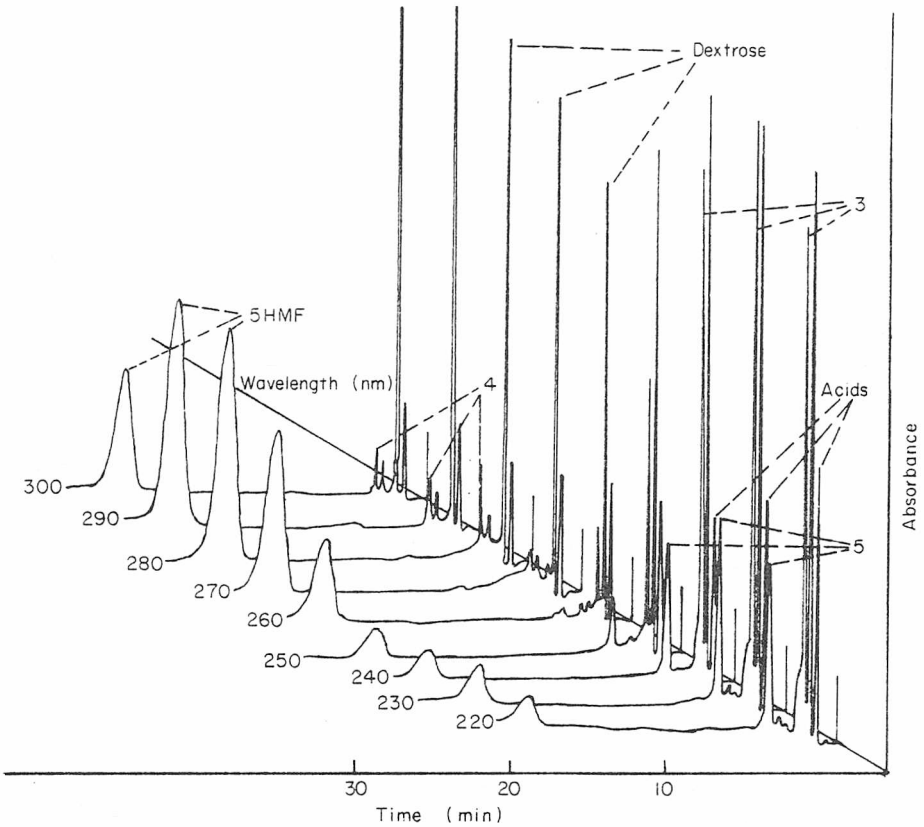

Fig. 1. Graphical representation of the chromatograms of degraded D-glucose solutions at several wavelengths. Peaks referred to in text are as labelled on figure. Chromatographic conditions: 120 × 4·6 mm Hypersil ODS column; solvent 0·05 M disodium hydrogen phosphate (pH 7), 20 μl injection volume.

In order to eliminate these inaccuracies the following method is suggested as a specific assay for 5HMF in autoclaved D-glucose solutions. While it is not suggested that this is an ideal quality control procedure for such solutions, it provides a datum from which comparison of the results obtained by the pharmacopoeial test and a specific 5HMF assay can be made.

Assay method for 5HMF

To 2 cm³ of degraded D-glucose test solution 25 μl of 0·1% w/v benzoic acid solution as internal standard was added. A 20 μl sample was injected on to the column using a valve and loop injector and the ratio of peak height of 5HMF and benzoic acid was determined. Figure 2 also shows a typical chromatogram obtained by such a procedure. The purity of the 5HMF peak was verified by its ultraviolet spectrum using the stopped flow technique.

A calibration curve was prepared using standard solutions of 5HMF of different concentrations. These were treated identically with respect to addition of internal standard and chromatography. This calibration curve resulted in the regression equation:

$$\text{Relative Peak Height } \frac{\text{5HMF}}{\text{BA}} = 988{\cdot}8\,(\text{5HMF}) + 4{\cdot}9 \times 10^{-2}\ R^2 = 0{\cdot}982$$

where (5HMF) is the 5HMF concentration in % w/v.

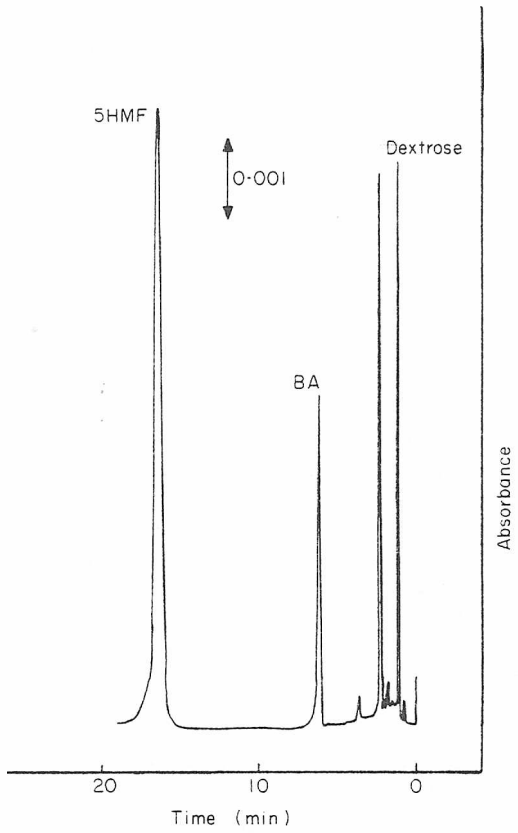

Fig. 2. Specimen chromatogram of degraded sample of D-glucose showing 5HMF peak and internal standard peak. Chromatographic conditions as in Fig. 1.

An aqueous eluent of 0·05 M disodium hydrogen phosphate (pH 7) was found to yield a consistent calibration curve independent of both column length and flow rate. In view of this constancy of calibration, the use of benzoic acid as internal standard appears adequate to normalize the chromatographic and detection variables and renders the routine use of 5HMF as a standard unnecessary. If it is required to alter the chromatographic solvent by the inclusion of the methanol or acetonitrile, recalibration would be necessary since 5HMF and benzoic acid retention times are differently affected by such organic modifiers.

Since the suggested assay method is highly specific for 5HMF and Fig. 1 indicates that a single absorption measurement at 284 nm is neither accurate in determining 5HMF alone nor is it adequate to measure all degradation products, the chromatographic assay was compared with the pharmacopoeial limit test at 284 nm as a method of determining 5HMF. Two series of measurements were made.

(i) A series of D-glucose solutions sealed in glass ampoules was heated at 120°C for various periods of time to produce different amounts of decomposition products including 5HMF. The solutions were analysed by the hplc method and were also subjected to the absorbance measurement at 284 nm after suitable dilution and an A (1%, 1 cm) value of 1316 (3, 4) used to estimate 5HMF concentration.

(ii) A series of Dextrose Injection samples autoclaved at 120°C for 30 min and having been

Table 1. Comparison between concentrations of 5HMF obtained chromatographically and spectrophotometrically in D-glucose samples decomposed at 120°C for various times

Time (h)	Absorbance	Concentration of 5HMF (% w/v × 10^3)	
		u.v.$_{284}$	hplc$_{254}$
0·5	0·112	2·13	0
1·5	0·173	3·28	1·15
2·0	0·223	4·33	2·30
2·5	0·320	6·08	5·00
3·0	0·440	8·35	7·20
3·5	0·565	10·70	10·1
4·5	0·895	17·0	17·0

Table 2. Comparison between concentrations of 5HMF obtained chromatographically and spectrophotometrically for Dextrose Injection 5% w/v samples autoclaved for 30 min at 120°C and subsequently stored for various times (a) after immediate opening and (b) resealing and storage for 3 weeks

Storage time (months)	Concentration 5HMF (% w/v × 10^3)			
	(a) Immediate		(b) Delayed	
	u.v.$_{284}$	hplc$_{254}$	u.v.$_{284}$	hplc$_{254}$
6	0·95	0·85	0·05	0
7	1·02	1·15	0·10	0
9	1·34	1·22	0·18	0
12	1·22	1·18	0·13	0
14	1·22	1·17	0·25	0
16	0·96	0·90	0·06	0
20	1·45	1·56	0·24	0
24	1·56	1·42	0·15	0
25	1·78	1·95	0·12	0
27	1·96	1·84	0·14	0

stored for periods of time from 6 to 27 months were also subjected to both assays. These samples were supplied by the Pharmacy Department of Aberdeen Royal Infirmary.

(i) The results of this investigation are shown in Table 1 and indicate that, at low concentrations of 5HMF, the hplc method yields consistently lower values for 5HMF than does the official limit test. At high 5HMF values, well above the limit for 5HMF and related products, the methods are in good agreement as an estimation of 5HMF concentration. The discrepancy at low levels is attributed to the contribution at 284 nm of the intermediate 4 shown in Fig. 1. At high values this is insignificant compared with the large 5HMF absorbance.

(ii) The measurement of impurity levels in terms of 5HMF on autoclaved samples of Dextrose Injection was felt to be a much more stringent test of the equivalence of the two

assay procedures. Because of this fact, measurements were made on each sample after an interval of 3 weeks, the sample being sealed between measurements. The results are shown in Table 2. The first estimate of 5HMF by both measurements yielded very comparable results. The hplc results are slightly lower on average as in the previous investigation, but the agreement is in general inferior to that obtained under controlled conditions of heating. This may be due to inaccuracies in one or both experimental procedures or may reflect variation among samples.

The repeated estimation of the impurity level, differing only from the first in that the containers had been opened, resealed and stored for a further 3 weeks, showed dramatic changes (Table 2). No 5HMF could be detected by the chromatographic method and a very small concentration of 5HMF was calculated by the single wavelength absorbance measurement. These results are interpreted as indicating that further decomposition of the 5HMF had occurred during storage following exposure to air. The pH of the Dextrose Injections was in the region of 3·7 and no appreciable alteration was observed on opening and storing. This fact, together with the appearance of two substantially unretained peaks on the solvent front of the chromatogram, indicated that acidic products had been produced in the solution.

The acidic nature of these further products and the wavelength dependence of their ultraviolet absorption was determined chromatographically using an eluent of 0·05 M disodium hydrogen phosphate buffer solution which was also 10 mM in tetrabutylammonium sulphate as ion-pairing agent. The results are shown in Fig. 3 in which two completely resolved and retained peaks, A and B, are observed. These acids are seen to have clearly defined maxima in the region of 250 nm and show minimal absorbance at 284 nm.

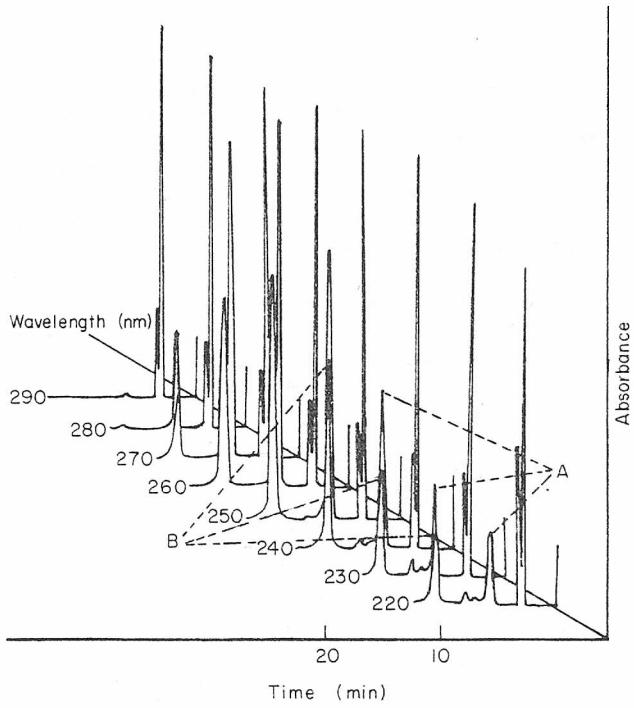

Fig. 3. Graphical representation of the chromatograms of autoclaved Dextrose Injection at several wavelengths after exposure to air and storage. Conditions as in Fig. 1 with solvent containing 10 mM tetrabutylammonium sulphate. Peaks A and B as referred to in text.

These observations explain the vastly different results obtained at different times for the autoclaved dextrose samples and further emphasize the quite erroneous results which can be obtained by the single absorbance measurement limit test. This may be of relevance in the quality control situation where the propensity for further reaction following exposure to air is not appreciated.

While the acidity of such solutions is controlled by both the British and European Pharmacopoeias, it would appear from the above observations that the quality control of autoclaved dextrose solutions could be obtained more rationally using a chromatographic method based on ion-pairing hplc, which would detect and quantify all appreciable impurities produced during autoclaving using the conventional 254 nm wavelength of detection. Alternatively, a better wavelength of absorption for a single wavelength limit test would be 250 nm, at which wavelength the possible acidic products and also intermediates would be measured while the 5HMF would be detected at reduced sensitivity.

Further work is in progress to examine the kinetics of the suggested oxidation process and the inter-relationships among the various intermediates and products. This will be the subject of a further communication together with identification of the acidic products.

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INVESTIGATION OF THE EFFECT OF ADDED ORGANIC AMINE ON THE CHROMATOGRAPHY OF TRICYCLIC ANTIDEPRESSANT DRUGS USING REVERSED-PHASE CHROMATOGRAPHY ON OCTADECYLSILICA WITH SODIUM LAURYL SULPHATE AS PAIRING ION

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SUMMARY

The chromatography of a group of tricyclic antidepressant drugs has been investigated using reversed-phase chromatography on ODS Hypersil with sodium lauryl sulphate as pairing ion both in the presence and absence of various organic amines. The amines investigated ranged in hydrophobicity from methylammonium to cetrimide. The general effect of such added amines is to decrease retention of basic solutes and to alter the selectivity of the stationary phase. The results are interpreted on the basis of the ion-exchange desolvation process previously proposed and optimised systems for the separation of clinically relevant solute pairs in this drug class are demonstrated.

INTRODUCTION

Currently, the most widely used high-performance liquid chromatographic (HPLC) mode is that of reversed phase using chemically bonded stationary phases with octyl- and octadecylsilanes being far the most commonly used modifiers of the silica surface[1]. Such stationary phases, together with suitable choice of aqueous mobile phase incorporating methanol or acetonitrile as organic modifier, will produce retention and usually excellent separation in most chromatographic problems involving electrically uncharged solutes.

The retention of ionogenic species can be achieved in the case of acidic solutes by control of pH[2,3]. Ion suppression, at low pH, will often allow chromatography of weak acids in the undissociated state. For basic solutes ion suppression is less advantageous due to deterioration of the silica support material at high pH[4,5].

Both types of electrolyte have been successfully chromatographed by addition of hydrophobic pairing ions, opposite in charge to the solute, to the mobile phase[6–9].

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In such cases the pH is adjusted so that the solute species is completely in the ionised condition. This procedure has been studied by very many workers and various terms have been coined to describe the processes leading to retention and separation[6–12]. In spite of the confusion produced by terminology, this technique has augmented the ion suppression method for the chromatography of acids and has extended chromatographic possibilities to strong acids where the pH required for complete suppression would be so low as to adversely affect the silica support[6]. Its major application has been to the chromatography of organic bases at low pH. Using such systems the chromatographic conditions can be controlled extensively by modification of the mobile phase, in particular the type and concentrations of pairing ion and associated counter ion used[13]. Recently the variation of retention and separation of several solutes has been shown to be complex with respect to mobile phase pairing ion concentration; the capacity factors of all solutes studied showing a maximum with pairing ion concentration. These results have been shown to be general for groups of both acidic and basic solutes and have been interpreted on a quantitative basis by an ion exchange model which also involves desolvation on the silanised silica surface[8,9]. On this model, counter ion concentration, *i.e.*, an ion of similar charge to that of the solute, is also involved in the retention equilibrium. It has been shown that the effect of increasing ionic strength and thus counter ion concentration has the effect of reducing retention in such systems[13,14]. Previous work on such counter ion dependence has been limited to inorganic counter ions where interaction is largely confined to the mobile phase.

The literature contains several reports of organic amines being added to the mobile phase during the chromatography of basic drugs, in particular the tricyclic antidepressant group of bases[4,15–18]. The effect of such addition is generally to reduce tailing of eluted peaks. This has been explained on the basis of masking active silanol groups[19] or of suppressing dissociation of ion pairs in the organic phase[17,18]. Most of the data on such separations have been obtained in liquid–liquid systems in which higher alcohols have been used to modify the C-18 or C-8 surfaces. Little information is available on the effect of such amine addition on the chromatography of basic drugs on modern systems involving an octadecylsilica surface without the addition of higher alcohols. Neither has there been any systematic study as to the relative effectiveness of different organic bases in improving the chromatography of basic solutes.

Because of the clinical importance of the tricyclic antidepressant drugs together with the poor chromatography generally associated with these compounds[20], they are used as a model set of compounds in the present investigation. It is intended to examine the effect of several amines and quaternary ammonium salts on the chromatography of the major drugs in this group and to attempt to explain the mode of action in the light of the ion exchange desolvation model of hydrophobic pairing ion retention previously proposed[8,9].

EXPERIMENTAL

Chromatography was carried out using a variety of equipment including Altex (Model 110A) and Waters Associates (M6000A) constant flow pumps. Detection at 254 nm was by Cecil (CE2012) and Pye Unicam (LC3) detectors. The wavelength of

measurement was not optimised in this study. Injection was by a Rheodyne 7125 valve fitted with 20- or 100-μl loops. Columns of conventional design and incorporating Swagelok fittings were either 100 mm or 70 mm long (4.6 mm I.D.) slurry packed at 600 bar with 5-μm ODS Hypersil (Shandon Southern Products). Retention time data were measured directly from chromatograms as recorded on a Servoscribe potentiometric recorder.

The tricyclic antidepressant drugs amitriptyline, nortriptyline, imipramine, desipramine, doxepin, maprotiline, mianserine, nomifensine, protriptyline, trimipramine, clomipramine and dothiepin were kindly donated by their manufacturers. The drugs were used as the hydrochlorides at a concentration of 50 μg cm^{-3}. Sodium lauryl sulphate (SLS), dimethylamine (DMA) and triethylamine (TRIEA) were obtained from Fisons and used as supplied. Methylammonium chloride (META), trimethylamine (TRIMA) and propylamine (PPLA) were obtained from BDH and tetramethyl-(TMA), tetraethyl-(TEA) and tetrabutylammonium (TBA) bromides were obtained from Aldrich. Cetrimide (CETA) was obtained from ICI Pharmaceuticals. Water used in chromatography was distilled before use and acetonitrile (HPLC grade) was obtained from Rathburn Chemicals and Fisons. All other reagents were of AnalaR or similar grade.

RESULTS

The separation obtained among twelve tricyclic antidepressant drugs is summarised in Fig. 1, which shows the variation of capacity factor, k', with the mobile phase concentration of sodium lauryl sulphate used as highly adsorbed pairing ion. While other pairing ions of lower hydrophobicity would affect the magnitude of the k' values obtained, previous work indicated that no substantial improvement in resolution could be obtained[9]. Fig. 1 shows that the capacity factors go through the expected maxima and that for most solutes maximum k' and thus optimum separation is obtained at a SLS mobile phase concentration of 80 mM. While considerable separation is obtained among several of the test compounds at the maximum in k', the clinically relevant separations, namely between imipramine and desipramine and between amitriptyline and nortriptyline, are not adequate. The separations achieved are further demonstrated in Fig. 2 which shows representative chromatograms of selected groups of drugs from among the compounds shown in Fig. 1. Fig. 2 indicates that the addition of pairing ion alone to the mobile phase is adequate to separate certain but not all compounds in the class. It also indicates that good chromatographic peak symmetry is achieved with little evidence of the peak tailing problems claimed to be associated with such compounds[17,18].

In order to evaluate the effect of counter ion type and concentration, various concentrations of different organic base salts, ranging in hydrophobicity from methylammonium chloride to cetrimide, were added to the mobile phase of 80 mM SLS in acetonitrile–buffer (50:50). Fig. 3 shows that the effect of such counter ion addition was general for a given test compound, namely to decrease the capacity factor. This effect was found to be general for all other compounds in the series but the extents of the decrease differed. Fig. 3 also indicates that the effect is greater the greater the apparent hydrophobicity of the added counter ion. The inverse relationship between k' and counter ion concentration previously noted for inorganic counter ions and

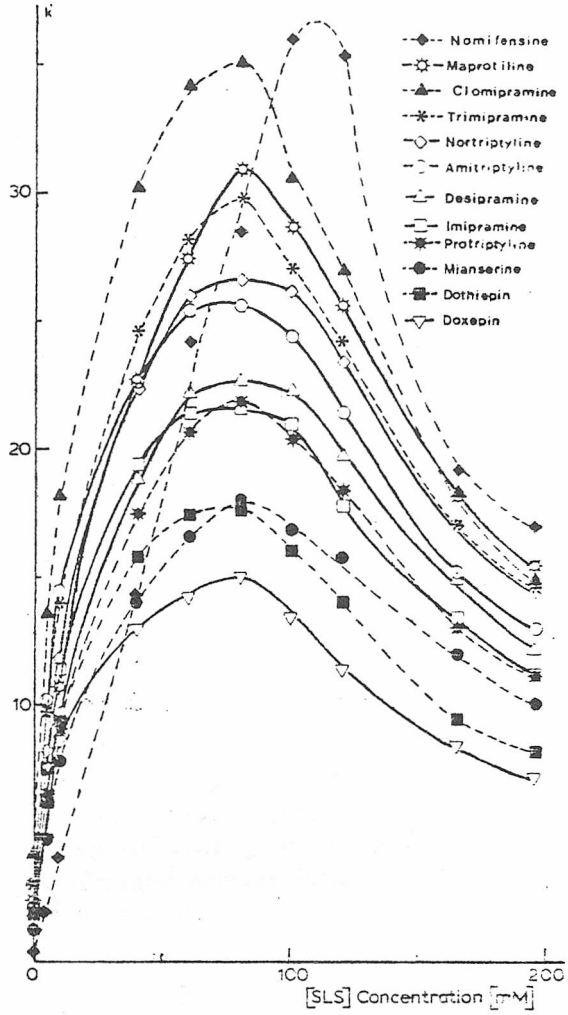

Fig. 1. Plots showing the variation of capacity factor, k', with mobile phase pairing ion (SLS) concentration for twelve tricyclic antidepressant drugs. Chromatographic conditions: column, 100 × 4.6 mm; mobile phase, acetonitrile–10 m*M* sodium dihydrogen phosphate (50:50) at pH 2; stationary phase, 5-μm ODS Hypersil; flow-rate 2.0 cm^3 min^{-1}.

predicted by the ion-exchange interpretation of ion pairing is not apparent from the present work with hydrophobic counter ions. It is also noted that while peak sharpening occurs, it is as a consequence of the decreased retention time. No real increase in plate number is obtained as a result of organic counter ion addition. Of greater significance is the effect of organic counter ion on the resolution between the pair amitriptyline and nortriptyline as shown in Fig. 4. The effect of adding amine to the mobile phase is to increase the resolution for this pair of compounds. The improvement is most marked in the case of cetrimide and least for methylammonium chloride. The effect of inorganic counter ion was observed to be minimal. These effects were identical for the imipramine–desipramine pair of compounds.

Although cetrimide shows by far the most noticeable effect on resolution both

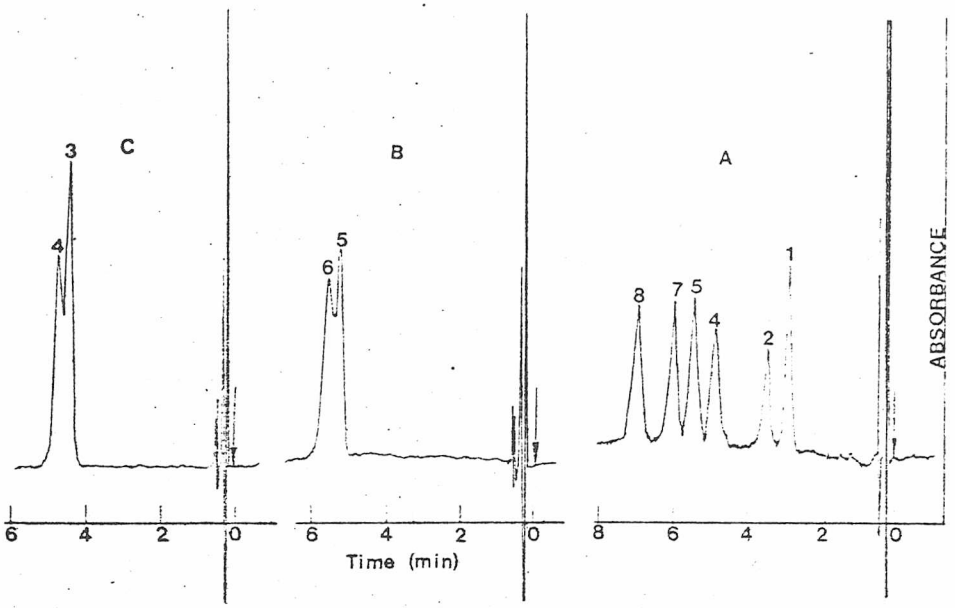

Fig. 2. Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs. Chromatographic conditions: column, 70 × 4.6 mm; mobile phase as in Fig. 1 with SLS concentration 80 m*M*. Compounds: 1 = doxepin; 2 = mianserine; 3 = imipramine; 4 = desipramine; 5 = amitriptyline; 6 = nortriptyline; 7 = trimipramine; 8 = clomipramine.

in the degree of resolution obtainable and in the minimum concentration required to achieve this, the retention times become very short and thus unacceptable for application to assay situations. Tetrabutylammonium (TBA) ion provides a better compromise between retention time and resolution for the above pair of compounds. Representative chromatograms are shown in Fig. 5, indicating the separations that can be achieved among selected members of the tricyclic antidepressant group of compounds by the addition of 5 m*M* TBA to 80 m*M* SLS. Comparison of Fig. 5 with Fig. 2 shows that although retention times are shorter, the peaks are considerably sharper and in fact measured plate numbers are comparable. It is also observed that resolution among the compounds has changed and that compounds previously separable without addition of added counter ion are now unresolved and *vice versa*. Complete data for the retention of all twelve compounds as a function of mobile phase pairing ion concentration in presence of a fixed TBA concentration of 5 m*M* are shown in Fig. 6. The maximum in k' with pairing ion concentration is still apparent but the relative magnitude of the capacity factors at the maximum is radically altered.

DISCUSSION

Previous explanations of the effect of added amine during the separation of basic drugs have suggested that the improvement in chromatographic behaviour is either as a result of decreased tailing due to the inactivation of unreacted silanol groups or alternatively, as a result of the suppression of secondary equilibria, namely the dissociation of ion pairs desolvated on the C-18 surface. Both of these expla-

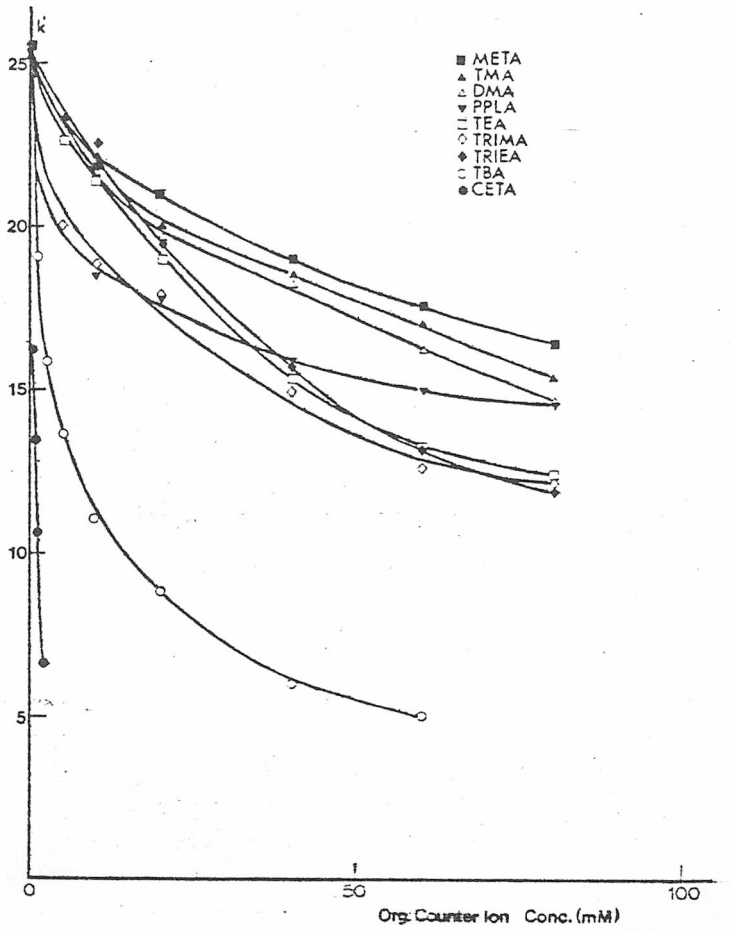

Fig. 3. Plots showing the variation in capacity factor, k', for amitriptyline as a function of various added counter ion concentrations. Chromatographic conditions as in Fig. 2, using a 100 × 4.6 mm column.

nations would require that the effect be general for all solutes and would result in a measurable increase in column efficiency for all basic solutes.

Any increase in efficiency in such systems involving added amine would be as a result of an increased rate of mass transfer. The mass transfer coefficient, measured as the slope of the HETP *vs.* mobile phase linear velocity curve at high linear velocities, was compared in presence and absence of 5 m*M* TBA for four solutes in a mobile phase of acetonitrile–water containing 80 m*M* SLS. Fig. 7 shows such plots for amitriptyline. No significant alteration in gradient was observed on addition of amine for any of the four solutes. This was taken as indicating constancy in the mass transfer rate and as further verifying the lack of real increase in column efficiency.

While the efficiency of the column is unaffected by addition of organic counter ion, resolution among the different solutes is markedly altered. The quantities contributing to resolution among any pair of compounds are included in the equation[21]

$$R = \frac{1}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{k'}{1 + k'}\right)N^{\frac{1}{2}}$$

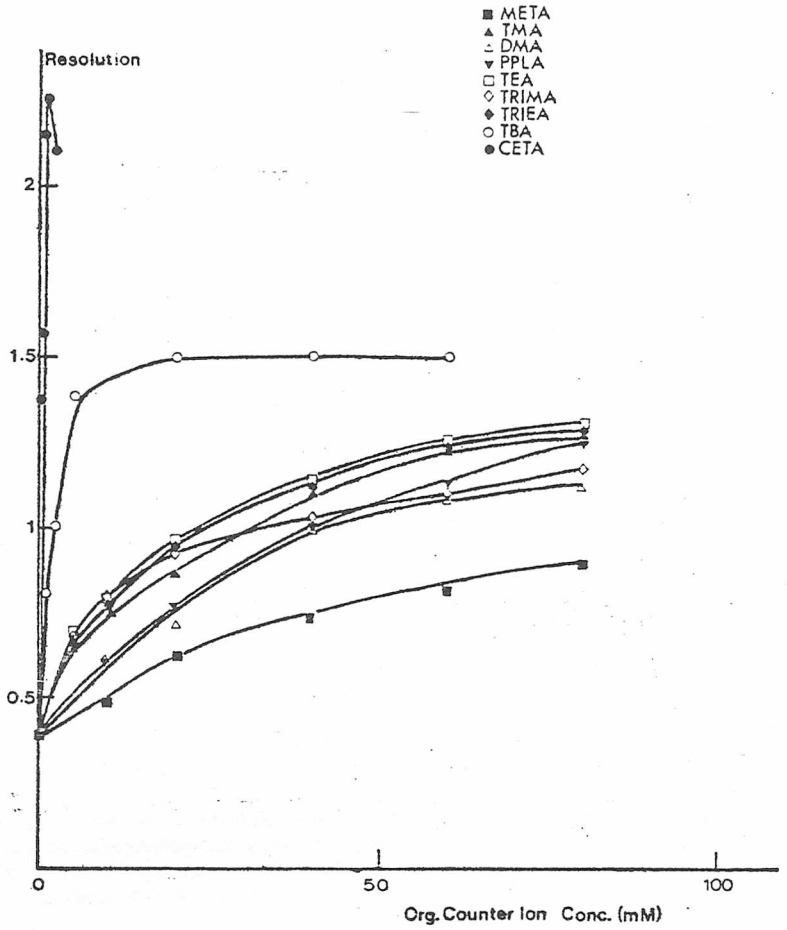

Fig. 4. Plots showing resolution between amitriptyline and nortriptyline as a function of various added counter ion concentrations. Chromatographic conditions as in Fig. 3.

where α represents the relative retention of the pair of compounds, k' the larger capacity factor and N the measured number of theoretical plates. In the present investigation, N is constant and the effect of added amine is to reduce k' so that any increase in R would appear to be as a consequence of a change in the selectivity term $(\alpha - 1)/\alpha$. The variation of $(\alpha - 1)/\alpha$ with concentration of added counter ion is shown in Fig. 8 and the overall shape of the plots appears to parallel that of the resolution as shown in Fig. 4. The ion exchange desolvation model formulated for hydrophobic pairing ions on octadecylsilica surfaces using aqueous mobile phases[8] can be used to explain the present findings.

This model represents retention of a basic solute in a completely ionised form by a hydrophobic anionic pairing ion adsorbed to an equilibrium extent on the C-18 surface. The basic equilibrium is one of ion exchange between the solute and the counter ion reinforced by desolvation of the solute on the C-18 surface. In the present investigation the hydrophobic nature of the counter ions will modify the behaviour from that observed with inorganic counter ions such as buffer salts, the interaction of which with the stationary phase pairing ion is purely electrostatic. When counter ions

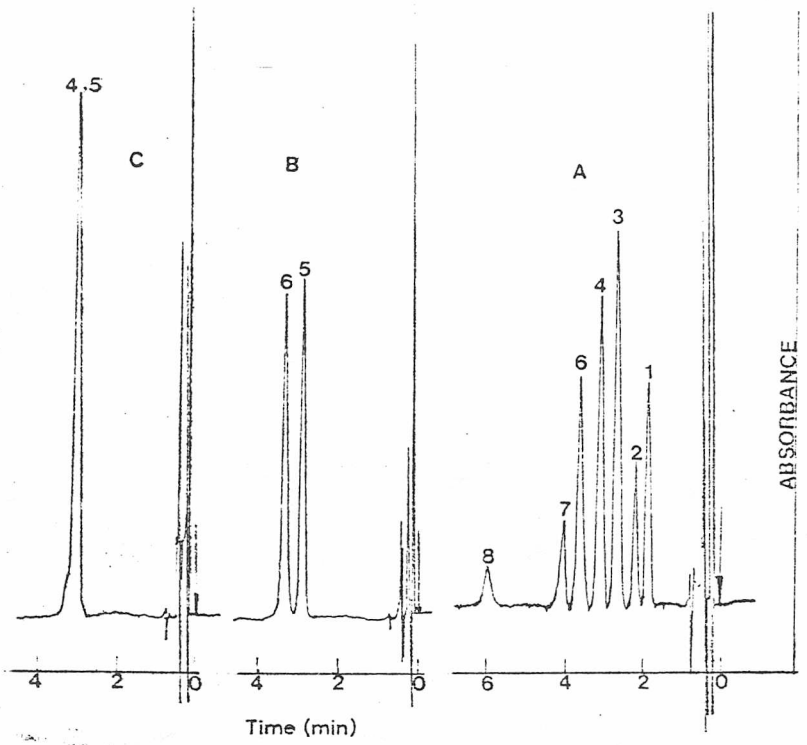

Fig. 5. Representative chromatograms showing complete and incomplete resolution obtained among certain tricyclic antidepressant drugs in presence of added organic counter ion. Chromatographic conditions as in Fig. 2 with the addition of 5 m*M* TBA to the mobile phase. Compounds: 1–6 as in Fig. 2; 7 = maprotiline; 8 = nomifensine.

differing in hydrophobic character are used, the counter ion, as well as being bound to the pairing ion by coulombic forces, is also desolvated on the adjacent C-18 surface. This equilibrium situation is identical in nature with the transient equilibria, producing retention of any hydrophobic solute. For retention of a solute to be achieved when organic counter ions are employed, not only must ion exchange occur between solute and counter ion, but the counter ion must be resolvated into the mobile phase. Thus, the more hydrophobic the counter ion, the more difficult will the resolvation process be and the greater will be the reduction in retention for a given concentration of added counter ion. Fig. 3 is interpreted on this basis as representing the effect of gradual replacement of inorganic counter ion by organic amine. The equilibrium reaction producing retention in the situation involving organic counter ion may be represented as before by the ion exchange reaction

$$(P^-C^-)_{org} + A^+_{aq} \rightleftharpoons (P^-A^-)_{org} + C^+_{aq} \qquad (1)$$

where $(P^-C^-)_{org}$ represents the adsorbed pairing ion together with its associated counter ion and A^- represents the fully ionised solute. The equilibrium constant, K_{IE}, will depend upon which species predominates as the counter ion C^+. That is, K_{IE} will vary from a maximum when the organic counter ion concentration is negligible to a

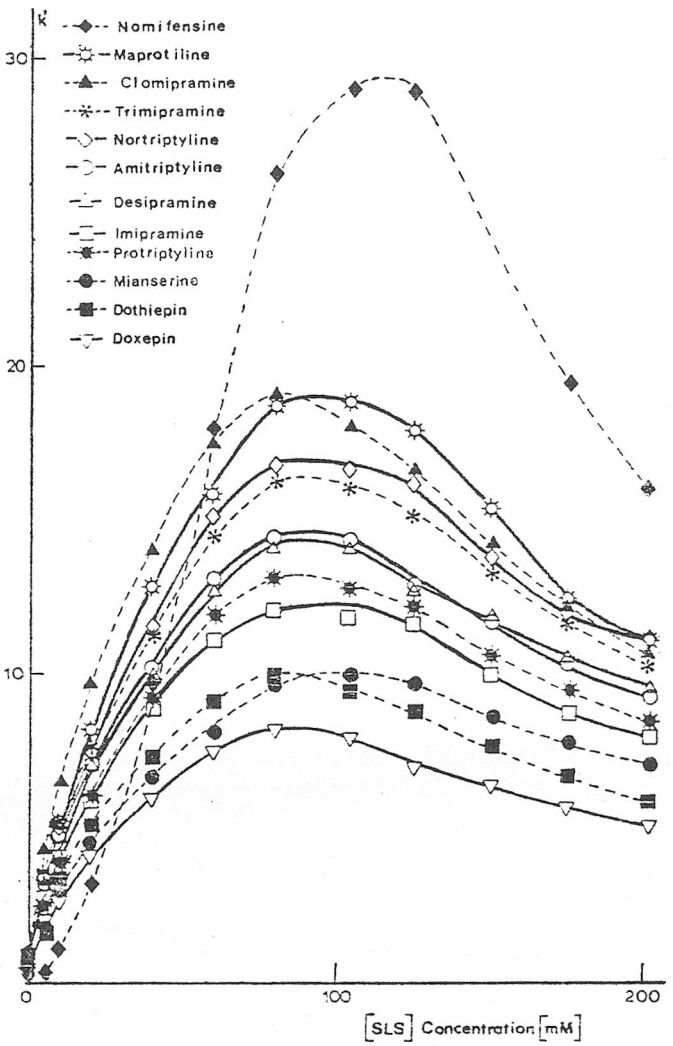

Fig. 6. Plots showing variation in k' with mobile phase pairing ion (SLS) concentration for antidepressant drugs. Chromatographic conditions as in Fig. 1 with the mobile phase modified to include 5 mM TBA.

minimum when all of the inorganic counter ion has been replaced by the added amine. Thus two limiting forms of the above equilibrium may be written:

$$(P^-Na^+)_{org} + A^-_{aq} \rightleftharpoons (P^-A^+)_{org} + Na^-_{aq} \ (K_{IE} \text{ large}) \quad (2)$$

$$(P^-TBA^+)_{org} + A^+_{aq} \rightleftharpoons (P^-A^+)_{org} + TBA^+ \ (K_{IE} \text{ small}) \quad (3)$$

At intermediate conditions, where not all of the sodium has been replaced due either to the limited hydrophobicity of the added amine, or to its low concentration, the value of K_{IE} will be intermediate between the two extremes.

The equation derived previously relating capacity factor to adsorbed pairing

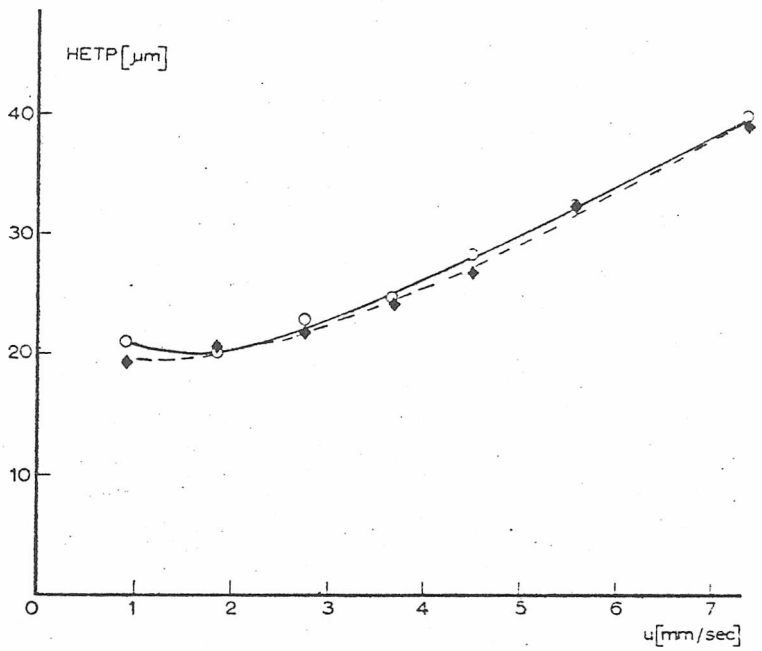

Fig. 7. Plot showing variation of HETP with linear mobile phase velocity for amitriptyline. Chromatographic conditions: O—O, as in Fig. 2, *i.e.*, with no added counter ion; ◆ ---◆, as in Fig. 5, *i.e.*, in presence of 5 m*M* TBA.

ion and aqueous counter ion concentration[8,9] will still apply for the condition where the counter ion concentration is held constant, *i.e.*:

$$k' = \frac{1}{V_m}\left(A_s K_1 - K_1 [P^- C^+]_{org} A_p + K_2 K_{IE} \frac{[P^- C^+]_{org}}{[C^+]_{aq}} - K_2 K_{IE} A_p \frac{[P^- C^+]^2_{org}}{[C^+]_{aq}}\right) \quad (4)$$

Eqn. 4 represents an improvement over the purely ion exchange processes represented above in that the ion exchange and desolvation tendency of a particular solute is represented by a combined constant K_2K_{IE}. The term K_1 refers to the desolvation constant of the solute in the non-ion pairing situation and will be small in comparison with the K_2K_{IE} term when separation is obtained. A_s and A_p are the areas of the stationary phase and pairing ion on a molar basis respectively, and V_m the void volume of the column.

This form of equation is seen to apply both in the situation of pairing ion and buffer only for all the tricyclic antidepressant drugs investigated as shown by Fig. 1 and also for the case of a fixed concentration of TBA as organic counter ion in presence of SLS pairing ion as shown in Fig. 6. In this equation in the case of organic counter ion the K_2K_{IE} term will have the increased significance that it will reflect not only the desolvation of a given solute subsequent to ion exchange but it will provide an estimate of the desolvation tendency of a solute displacing an already desolvated organic counter ion. This is seen as providing an additional parameter of selectivity in such systems and may account for the alteration in elution order in Fig. 6 compared with Fig. 1. It is emphasised that while in this case the selectivity alteration has been

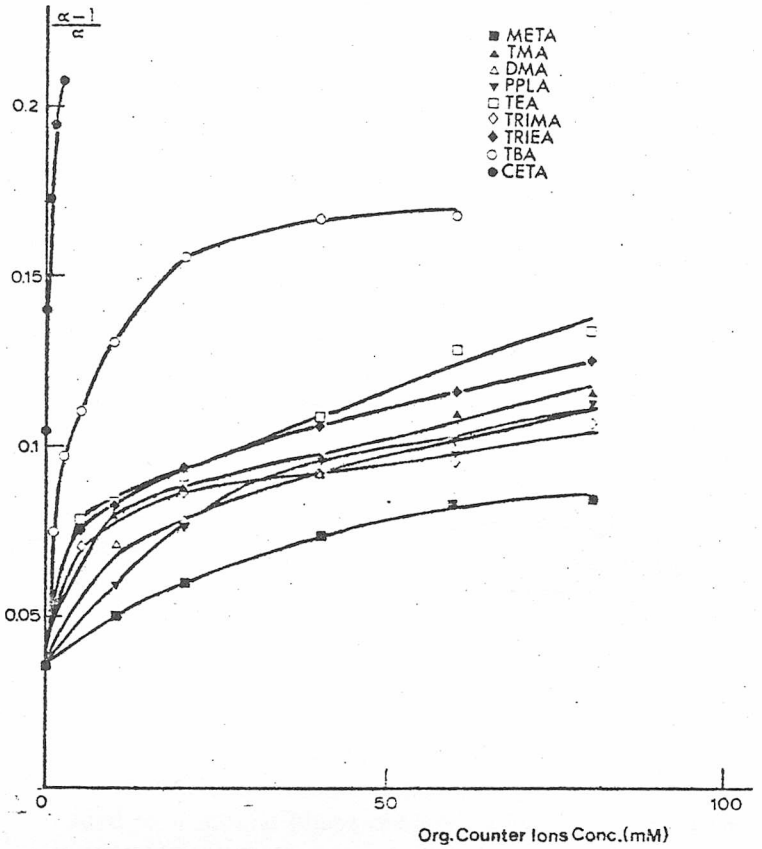

Fig. 8. Plots of the variation of selectivity $(\alpha - 1)/\alpha$ between amitriptyline and nortriptyline as a function of different organic counter ion concentrations. Chromatographic conditions as in Fig. 3.

to improve the resolution for the pairs desipramine and imipramine and amitriptyline and nortriptyline, its effect is also to reduce the resolution between other pairs of compounds as shown in Fig. 5C for desipramine and amitriptyline.

The applicability of the above equation on a quantitative basis for the above pairs of compounds can be assessed by determining the K_2K_{IE} constants for each compound in presence and absence of organic counter ion. The K_2K_{IE} constants were evaluated by fitting the curves for the appropriate compound as shown in Figs. 1 and 6 to the above equation by the method of least squares using the Hooke software system previously employed[8,9]. $[P^+C^+]_{org}$ values were obtained by interpolation of values from previous isotherms[9] measured at 30% and 60% acetonitrile concentrations and it is assumed on this model that the area available for desolvation of solutes will be unaffected by the presence of the more bulky organic counter ion. If the equation is applicable to such systems involving added organic counter ion, the numerical values of K_2K_{IE} should be seen to decrease in the presence of organic counter ion. In addition the separation factors, α, which can be measured by direct observation of the relative retention times between pairs of compounds should be identical with the ratio of the calculated K_2K_{IE} values for that pair of compounds obtained by the above curve fitting procedure. The results of such a comparison are shown in

TABLE I

OBSERVED SEPARATION FACTORS AND CALCULATED RATIOS OF ION EXCHANGE DESOLVATION CONSTANTS, K_2K_{IE}, DERIVED FROM EQN. 4 IN PRESENCE AND ABSENCE OF ORGANIC COUNTER ION FOR SELECTED PAIRS OF COMPOUNDS

Compound	*SLS (80 mM)*			*SLS (80 mM) + TBA (5 mM)*		
	K_2K_{IE}	K_2K_{IE} *ratio*	α	K_2K_{IE}	K_2K_{IE} *ratio*	α
Imipramine	0.179	1.04	1.05	0.0955	1.188	1.129
Desipramine	0.185			0.114		
Amitriptyline	0.208	1.06	1.04	0.109	1.250	1.123
Nortriptyline	0.219			0.136		
Desipramine	0.185	1.12	1.13	0.114	1.05	1.03
Amitriptyline	0.208			0.109		

Table I. It is seen that the expected decrease in K_2K_{IE} is observed and that good agreement is obtained between α values and K_2K_{IE} ratios calculated both for the situation where resolution has been improved and for that in which resolution has been decreased.

CONCLUSIONS

The present investigation using the closely related group of tricyclic antidepressant drugs as a model system indicates that the action of an organic amine when added to a mobile phase containing hydrophobic anionic pairing ion is that of a counter ion involved in an ion exchange desolvation process. Such species, because of their desolvation on the C-18 surface, act to reduce capacity factors for all basic solutes. They may also provide an additional degree of selectivity over that observed in such systems in absence of added amine. The effect may be utilised to increase resolution among certain compounds but may also act to reduce resolution among others. It has been found that the systems discussed can provide the required selectivity and sensitivity for the measurement of these drugs in serum at therapeutic levels. The quantitative results of such an assessment will be the subject of a future communication.

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