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THE INVESTIGATION OF ION-EXCHANGE PROPERTIES OF NOVEL ZWITTERIONIC AND AMPHOTERIC STATIONARY PHASES AND THEIR APPLICATION TO THE SEPARATION OF INORGANIC AND ORGANIC IONS

Ekaterina Pavlovna Nesterenko, M.Sc.

Student No.: 54162459.

Under the supervision of Prof. Brett Paull.

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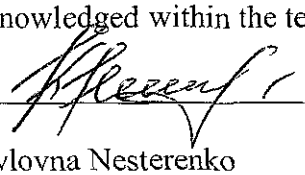
DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy is entirely my own work and has not been taken from the work of others, save and to the extent that such work has been cited and acknowledged within the text of my work.

Signed: 

Ekaterina Pavlovna Nesterenko

Student Number: 54162459

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ABSTRACT

Over recent years development of ion chromatography (IC) has focused on new phase technologies to simultaneously increase efficiency and vary selectivity. To increase selectivity it is necessary to develop new selective ion-exchangers by varying the nature of functional groups and the matrix of the stationary phase. Zwitterionic ion-exchangers, where positive and negative charges are located in close proximity, exhibit potential for selectivity optimisation in IC due to variation of the ratio of electrostatic attraction/repulsion forces between analyte ions and ion-exchange groups. The main advantage of zwitterionic ion-exchangers is the possibility of their use for the simultaneous separation of cations and anions and the use of diluted eluents, which significantly increases the sensitivity of detection and can contribute to the increased efficiency.

Here various reversed-phase sorbents dynamically coated with *N*-(dodecyl-*N,N*-dimethylammonio)alcanoates were produced and characterised. The ion-exchange properties of these new phases were investigated and the retention mechanism of ions on these stationary phases elucidated. From investigations into the effect of pH, concentration, nature and charge of the eluent cation on the retention of anions, it was shown that separation of anions occurs due to chaotropic interactions with quaternary ammonium groups, shielded by external weak carboxylic acid groups, which themselves interact with eluent cations according to increasing charge. These effects were exploited to demonstrate a previously unreported cation charge gradient approach to anion separations, resulting in considerable reductions in retention times whilst maintaining resolution. In addition, the new phases were used with a novel combined triple eluent concentration, pH and flow gradient technique for the simultaneous separation of 18 nucleotides, nucleosides and nucleobases and the method developed was applied to the separation of nucleic acids precursors in yeastolate samples. An investigation into the retention of transition metal cations was also made and it was shown that cations could be simultaneously separated with inorganic anions, as anionic complexes with the eluent anion, on the new zwitterionic phase. This simultaneous separation of inorganic anions and cations was shown and optimised conditions were applied to the separation of anions and cations in a natural water sample. Finally, the application of short monolithic zwitterionic columns for anion separations was investigated, with the separation of 6 anions obtained under flow gradient conditions on a 4 mm - long column coated with *N*-(dodecyl-*N,N*-dimethylammonio)undecanoate and an application to the analysis of a saline sample is shown.

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A LIST OF PUBLICATIONS AND CONFERENCE PRESENTATIONS

Publications:

1 **Ekaterina P. Nesterenko**, Pavel N Nesterenko, Brett Paull. *"Anion-exchange chromatography on short reversed-phase columns modified with N-(dodecyl-N,N-dimethylammonio)alcanoates*". Journal of Chromatography A **1178** (2008), 60-70.

2. **Ekaterina P. Nesterenko**, Celine Guivarc'h, Cepta Duffy, Brett Paull. "*Separation of nucleic acid precursors on an amphoteric surfactant modified monolith using combined eluent flow, pH and concentration gradient*", Journal of Separation Science, **30**, (2007), 2910-2916.
3. **Ekaterina P. Nesterenko**, Leon P. Barron, Pavel N. Nesterenko, Brett Paull. "*Flow gradient liquid chromatography using a coated anion exchange microcolumn*", Journal of Separation Science **29**, (2006), 228-235
4. Colman O Riordain, Leon Barron, **Ekaterina P. Nesterenko**, Pavel N. Nesterenko, Brett Paull. "*Double gradient chromatography using short monolithic columns modified with a long chained zwitterionic carboxybetaine surfactant.*" Journal of Chromatography A, **1109**, (2006), 111-119.

Oral presentations:

1. **Ekaterina P. Nesterenko**, Brett Paull. "*Selectivity and versatility of zwitterionic ion columns prepared using N-(dodecyl-N,N-dimethylammonio)alcanoates*". XIXth Annual International Ion Chromatography Symposium (IICS2006), 24th-27th September 2006, Pittsburgh, PA, USA, P.8.

Poster presentations:

1. **E.P.Nesterenko**, B.Paull. "*Separation of nucleic acid precursors on N-(dodecyl-N,N-dimethylammonio)undecanoate modified stationary phases* ", Analytical Research Forum (ARF2007), 16th -18th July 2007, Glasgow, UK. P05.
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3. **E.P.Nesterenko**, B.Paull. "*Anion exchange properties of reversed-phase sorbents coated with (N-dodecyl-N,N-dimethylammonio)alkanoates.*", Analytical Research Forum (ARF06), 17th -19th July, 2006. Cork, Ireland. P09.
4. **E.P.Nesterenko**, B.Paull. "*Anion-exchange properties of N-(dodecyl-N,N-dimethylammonio)alkanoate coated stationary phases* ", International congress on Analytical Sciences (ICAS 2006), 25-30 June 2006, Moscow, Russia.
5. **E.P.Nesterenko**. "*Anion-exchange properties of reversed-phase sorbents, coated with N-(dodecyl-N,N-dimethylammonio)alkanoates.*" International student conference Lomonosov 2006, 10th -12th April, 2006. Moscow, Russia.
6. **E.P.Nesterenko**, B.Paull. "*Anion-exchange properties of reversed-phase sorbents, coated with N-(dodecyl-N,N-dimethylammonio)alkanoates.*", 4th Biennal Conference on Analytical Sciences in Ireland. 11th-12th April 2006. Dublin Institute of Technology, Ireland. P.30.
7. **E.P.Nesterenko**, B.Paull. "*Anion-exchange properties of Gemini C_{18} column, coated with N-(dodecyl-N,N-dimethylammonio)undecanoate.*", Analytical Research Forum (ARF05), 18th -20th July, 2005. Plymouth, UK. P.42
8. **E.P.Nesterenko**. "*Anion-exchange properties of Gemini C18 column, coated with (N-dodecyl-N,N-dimethylammonio)undecanoate.*", International student conference Lomonosov 2005, 11th -13th April, 2005 Moscow, Russia. P.32

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A LIST OF ABBREVIATIONS MENTIONED IN THE TEXT

α	Selectivity
ζ	Electrokinetic potential
ψ	Electrostatic potential
A	Adenosine
Ade	Adenine
ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
ATP	Adenosine-5'-triphosphate
BSA	Bovine serum albumin
C	Cytidine
CDP	Cytidine-5'-diphosphate
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate
CHAPSO	3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate
CMC	Critical micelle concentration
CMP	Cytidine-5'-monophosphate
CTAB	Cetyltrimethylammonium bromide
CTAC	Cetyltrimethylammonium chloride
CTC	Cetylpyridinium chloride
CTP	Cytidine-5'-triphosphate
Cyt	Cytosine
DCTA	Diaminocyclohexanetetraacetic acid
DDAB	Didodecyldimethylammonio bromide
DDAPS	3-(*N*-dodecyl-*N*,*N*-dimethilammonio)propane-1-sulphonate
DDMAA	(Dodecyldimethylammonio)acetic acid
DDMAB	*N*-(dodecyl-*N*,*N*-dimethylammonium)butyrate
DDMAU	*N*-(dodecyl-*N*,*N*-dimethylammonium)undecanoate
DMAES	2-(dimethylamino)ethanesulphonic acid
DOSS	Dioctylsulphosuccinate
d_{pores}	Pore diameter, nm
DTPA	Diethylenetriaminopentaacetic acid
EDL	Electric double layer
EDTA	Ethylenediaminetetraacetic acid
EGTA	Eethyleneglycol-bis2(2-aminoethylether) – *N*,*N*,*N'*,*N'* – tetraacetic acid

EIC	Electrostatic ion chromatography
F	Flow rate, mL/min
G	Guanosine
GDP	Guanosine-5'-diphosphate
GMP	Guanosine-5'-monophosphate
GTP	Guanosine-5'-triphosphate
Gua	Guanine
I.D.	Internal diameter
IC	Ion chromatography
ICP-AES	Inductively-coupled plasma atomic emission spectroscopy
k	Capacity factor
Li-DS	Lithium dodecylsulphate
MeCN	Acetonitrile
MES	4-morpholineethanesulphonic acid
N	Efficiency
ODS	Octadecylsilica
PAR	Pyridylazoresorcinol
PCR	Post-column reaction
PDMDAA	Poly(dimethyldiallylammonium) chloride
PEVP	Poly(N-ethyl-4-vinylpyridinum bromide)
PHMG	Poly(hexamethyleneguanidinum)hydrochloride
RP	Reversed-phase
R_s	Resolution
S	Surface area, m^2/g
Thy	Thymine
TTA	Tetradecyltrimethylammonium
U	Uridine
UDP	Uridine-5'-diphosphate
UMP	Uridine-5'-monophosphate
Ura	Uracil
UTP	Uridine-5'-triphosphate
UV	Ultraviolet
V_{pores}	Pore volume, mL/g
ZIC	Zwitterionic ion chromatography

CHAPTER 1. ZWITTERIONIC STATIONARY PHASES IN ION CHROMATOGRAPHY

Ion chromatography (IC) has become the method of choice for the determination of inorganic cations and anions since its introduction by Small *et al.* in 1975 [1] and is now the most widely applied analytical method for the determination of the ion composition of aqueous samples. The main parameter, on which separation selectivity strongly depends, is the nature of the ion-exchange group and the sorbent. The variety of different matrices and surface ion-exchange groups facilitates the application of IC to solving a wide range of analytical problems. Most common ion-exchangers exhibit either anion-exchange or cation-exchange stationary phase properties. Many different methods for the determination of inorganic anions, cations of alkali, alkaline-earth, transition and rare-earth metals have been developed and a large number of reviews published [2-9].

A promising trend in IC phase development has been the emergence of zwitterionic ion-exchangers [3,10,11]. The presence on the stationary phase surface of oppositely charged functional groups imparts unique properties to these zwitterionic stationary phases. Compared to standard cation- or anion-exchangers, the retention of ions on the zwitterionic stationary phases depends not only on ion attraction towards oppositely charged groups, but also on repulsion from groups with the same charge. The attraction/repulsion forces ratio depends on the nature of the ion-exchange groups and their location. Thus, the repulsion from ion-exchange groups with the same charge as the analyte ion reduces diffusion inside the stationary phase and as a result increases the mass transfer rate, which can lead to an increase in separation efficiency. At the same time these weak ion interactions, resulting from the use of zwitterionic stationary phases also allow the use of diluted electrolytes or even water as a mobile phase, which can be important when non-specific detection (*e.g.* conductivity) is used.

One of the main interests in zwitterionic stationary phases is due to the possibility of their use for the simultaneous separation of cations and anions. This is especially important for water analysis, as this approach can both reduce analysis time and consumption of reagents. In the past for the simultaneous separation of cations and anions several approaches have been suggested:

1. The use of a chromatographic system with coupled anion- and cation-exchange columns [12-14];

2. Obtaining anionic complexes of cations and their simultaneous separation with anions on anion-exchangers [15,16];

3. The use of chromatographic columns, filled with the mixtures of polymer/silica based anion and cation exchangers, and stationary phases that contain both chemically bonded anion and cation ionogenic groups [17-21];

4. The use of weak cation-exchangers, where retention of cations occurs due to ion-exchange interactions and anion retention occurs due to ion-exclusion effect [22,23].

However, the above methods, with the exception of the latter, have not found wide application to-date, due to the inconvenience of methods utilising two columns, low reproducibility of mixed stationary phases, or the impossibility of obtaining anionic complexes of alkali metal cations.

1.1. Classification of zwitterionic stationary phases

Zwitterionic ion-exchangers are considered to include only those materials, where groups of both charges are present on a single particle, but not mixed-bed ion-exchangers where a mixture of cation- and anion-exchangers are used. Depending on the distribution of the oppositely charged groups the following types of zwitterionic exchangers can be identified:

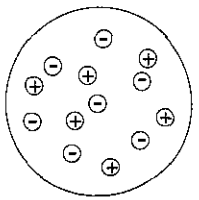
1. Polyampholyte stationary phases with chaotic distribution of oppositely charged groups in the whole volume of the ion-exchange particle or on its surface;

2. Pellicular resins, containing a layer of opposite charge on a charged core particle (microparticles, latex beads, polymer films and *etc.*);

3. Ion-exchangers, where oppositely charged groups are present in a single attached or immobilised molecule and form two oppositely charged layers.

Figure 1-1-1 shows schematic representations of each of the above categories.

1. Oppositely charged groups are chaotically distributed within the whole volume of the particle or its surface.

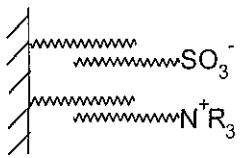


a. Organic and inorganic zwitterionic polymers.

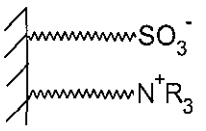
e.g. Zwitterionic polymers.
e.g. Amphoteric metal oxides.

b. Immobilised proteins.

c. Zwitterionic ion-exchangers with separately distributed groups on the surface.

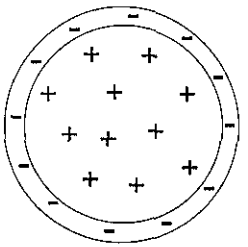


e.g. Stationary phases dynamically coated with cationic and anionic surfactants.

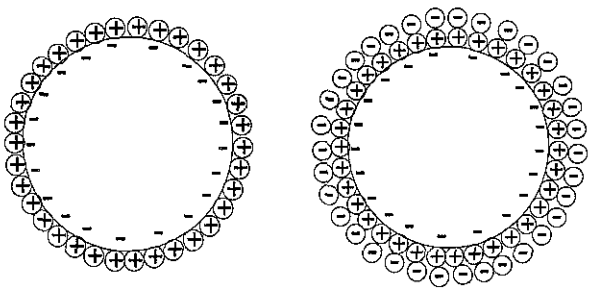


e.g Stationary phases with covalently attached cation- and anion-exchange groups.

2. Oppositely charged groups form two oppositely charged layers.



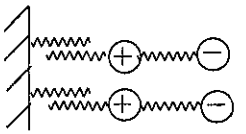
a. Centrally localised ion-exchangers.



b. Agglomerated ion-exchangers.

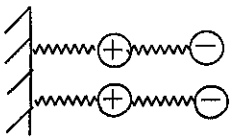
e.g. Stationary phases modified with polysaccharides or ionenes.

3. Oppositely charged groups are located in one molecule attached to the surface.



a. Dynamically adsorbed zwitterionic molecules.

e.g ODS dynamically coated with zwitterionic surfactants



b. Covalently bonded zwitterionic molecules.

e.g. Amino acid bonded silica.

Figure 1-1-1. Types of zwitterionic ion-exchangers in liquid chromatography

1.2. Zwitterionic ion-exchangers with oppositely charged groups distributed within the particle or on its surface

1.2.1. Polyampholyte ion-exchangers

Though many polyampholyte ion-exchangers (*e.g.* phenol-formaldehyde polycondensation type resins or silica based anion-exchangers, which contain acidic phenol or silanol groups) with differing ratios of cation- and anion-exchange groups have been synthesised, they have not found significant application in liquid chromatography. However, in recent years interest in this type of stationary phase has increased due to the development of polysulphobetaines and polycarboxybetaines [24-26] This type of polymer, as described by Perez *et al.* [27] was used for the synthesis of monolithic stationary phases for the separation of proteins [28].

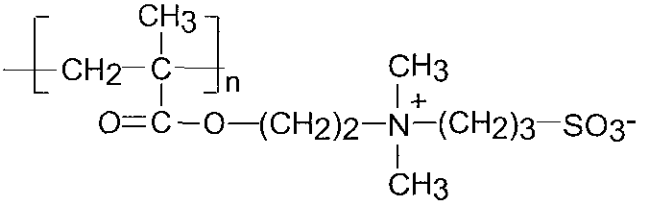


Figure 1-2-1. Structure of polysulphobetaine

This stationary phase exhibits strong retention for positively charged proteins, while neutral or negatively charged proteins elute in the dead volume, thus showing cation-exchange properties. This may be due to the polymer configuration and steric inaccessibility of anion-exchange groups for bulky molecules, or negatively charged molecules experiencing repulsion from sulphonate groups, which carry the same charge. At the same time, small inorganic anions can reach the quaternary ammonium groups and elute in the order: $Cl^- < Br^- < I^- < SCN^-$.

1.2.2. "Snake - cage" type stationary phases

Retardion or so-called "snake - cage" type resins were first synthesised in 1957 by Hatch *et al.* [29]. This type of stationary phase consists of poly(styrenedivinylbenzene) net with suitable ion-exchange groups attached ("the cage"), inside which exists an organic counter ion that can be polymerised to form linear chains of an oppositely charged polymer ("the snake"). The polymer chain cannot be removed from the polymer matrix due to strong electrostatic interactions and restricted mobility. As oppositely charged sites are neutralised by each other, improved

mechanical stability is achieved, which leads to reduced resin swelling and shrinking effects [30,31].

Originally, this type of stationary phase was developed for desalting non-ionic solutions due to its low affinity for non-electrolytes [32]. Therefore, it can also be used for separating unwanted ions from ionic substances or for removing acids following acid hydrolysis of proteins [31,33]. Unfortunately, this type of stationary phase has not been widely used for chromatographic separations [34], but was successfully used for ion preconcentration prior to further radiochemical detection [35,36].

In was shown that the Retardion AG11A8 resin (a commercial "snake - cage" resin) has the following affinity in pure water for anions:

$$SO_4^{2-} < CO_3^{2-} < I^- < Br^- < Cl^- < CH_3COO^- < SCN^- \approx ClO_4^-$$

and for cations:

$$K^+ < Na^+ < Li^+ < Zn^{2+} < Cu^{2+}.$$

It is interesting to note the low affinity of the resin towards sulphate and the relatively high affinity for acetate. The selectivity of Retardion AG11A8 for many metals ions in different solutions and varying pH was investigated by Dybczynski [30,35-38]. It was shown that apart from cations, such as Ag(I), Cs(I), Cd(II), Co(II), Cu(II), Mn(II), Zn(II), Ni(II), Cr(III), Fe(III), Ga(III), In(III), La(III), Retardion11A8 adsorbs oxoanions of Re(VII) and Mo(VI) from NH_4Cl solutions [30]. Zwitterionic properties of this stationary phase were used for the extraction of cations, capable of anionic complex formation, *e.g.* Zn(II) [36,37] and Cd(II) [35,37], from biological samples. It should be noted that ampholytes, such as amino acids, are less retained than salts formed from strong acid and strong bases, suggesting the absence of two-points of coordination (or "quadrupole" interactions) between the functional groups of the amino acid and the oppositely charged sites on the resin [39].

1.2.3 Amphoteric inorganic oxides

It is known that stationary phases, based on inorganic oxides can exhibit cation- or anion-exchange properties depending on the eluent pH. One of the most studied in this group of stationary phases is aluminium oxide. Alumina, like other hydrous oxides, has a very complex surface structure, which is dependent on pretreatment and chemical environment. It is suggested that a surface charge appears due to the dissociation of

surface =AlOH groups and dissociation of either hydrogen or hydroxide ions. In the acidic pH region alumina exhibits anion-exchange properties, while in the basic region it exhibits cation-exchange properties:

$$=Al^{\oplus} \xleftrightarrow{H^+} =Al-OH \xleftrightarrow{OH^-} =Al-O^{\ominus}$$

The isoelectric pH for alumina, which is dependent on buffer components and the type and treatment of the alumina, can vary from pH 3.5 (citrate) to 9.2 (carbonate), while its ion exchange capacity, which approaches 2 mequiv/g, is also very dependent on the analyte ion, the pH, and the type and pretreatment of the alumina [40].

In general, the change from an anion exchanger to a cation exchanger is a gradual one and occurs in the vicinity of the isoelectric pH. Rates of ion exchange, which are also pH, environment, analyte, and type of alumina dependent, are favorable and it is suggested that alumina under appropriate pH conditions functions as a monofunctional cation exchanger and a polyfunctional anion exchanger.

One of the most significant differences of alumina from polymer ion-exchangers is the anion-exchange selectivity:

$$CH_3COO^- < ClO_4^- < I^- < SCN^- < ClO_3^- < Br^- < NO_3^- < NO_2^- < Cl^- < BrO_3^- < ClO_2^- < C_6H_5COO^- < SO_4^{2-} < F^-$$

The elution order of halide anions corresponds with the increase of stability of these anion complexes with aluminium. Apparently, the formation of complexes can also contribute to the retention of other anions [41].

1 2.4. Stationary phases with attached cation- and anion-exchange groups

One of the easiest ways to prepare this type of zwitterionic ion-exchanger is the dynamic modification of a RP sorbent with a mixture of cationic and anionic surfactants. By varying the ratio of these surfactants it is possible to obtain a stationary phase with the required cation and anion capacity, and thus to regulate the selectivity of the stationary phase itself. Thus, octadecylsilica (ODS) treated with a mixture of octanesulphonate and tetrabutylammonium has been shown to give a stationary phase with zwitterionic properties [42]. This stationary phase was used to achieve the simultaneous separation of neutral, positively and negatively charged Pt(II) complexes. It is worth noticing that the capacity of this stationary phase was less than that of ODS

modified individually with either cationic or anionic surfactant, and to increase stability of the coating it was necessary to add surfactants to the eluent.

Recently, a number of studies on the separation of cations and anions have been carried out using cationic and anionic surfactants, such as cetyltrimethylammonium halide (CTAC, CTAB) [43-45], cetylpyridinium chloride (CTC) [46,47], didodecyldimethylammonio bromide (DDAB) [48-54], lithium dodecylsulphate (Li-DS) [55] and sodium dioctylsulphosuccinate (DOSS) [50,56] (for structures see Fig. 1-2-2).

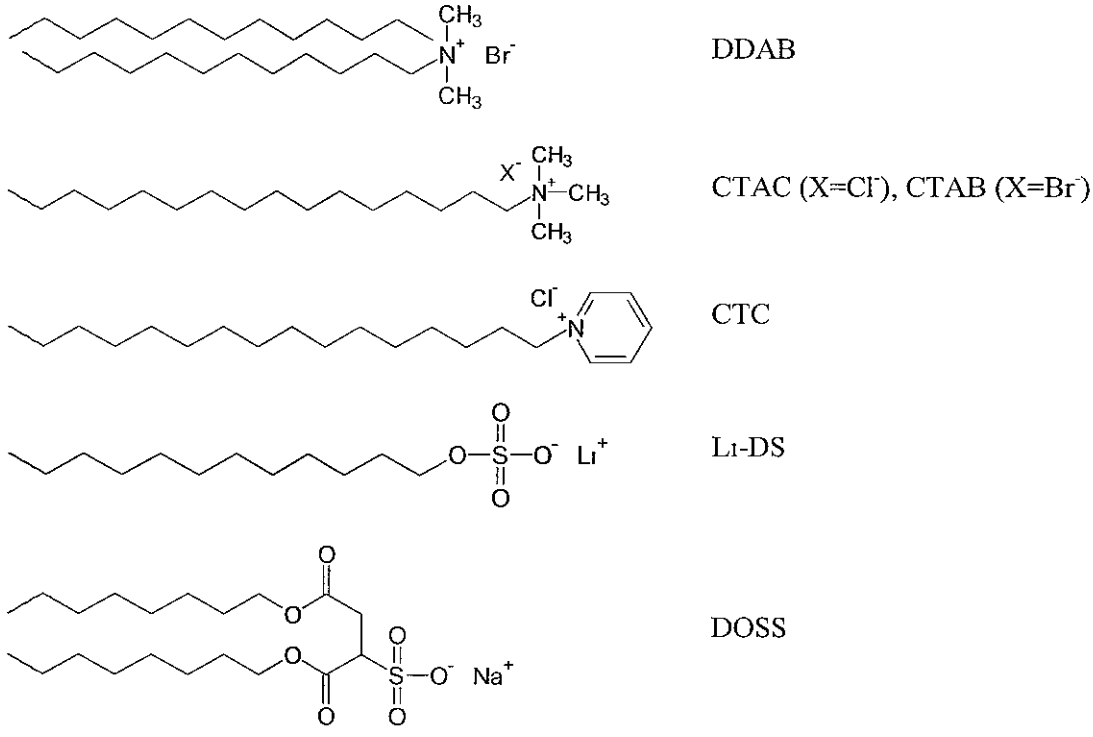


Figure 1-2-2. Structures of surfactants used for dynamic coating

However, in the majority of these investigations all surfactants were used individually for either cation or anion analysis. Thus, Hatsis *et al.* [49] achieved the separation of 7 anions in 30 seconds using DDAB as a RP monolithic column coating, with a phthalate eluent [57]; the same authors also obtained the separation of 8 common anions including, IO_3^-, Cl^-, NO_2^-, Br^-, NO_3^-, HPO_4^{2-} and SO_4^{2-}, in 15 seconds at a flow rate of 16 mL/min. RP columns coated with CTAC and CTAB were used for determination of nitrite and nitrate [44] and bromide [53] in sea water, and monolithic ODS columns coated with CPC were applied for determination of chromate [47] in sea water in less than 30 seconds. The anionic surfactant Li-DS was used for coating monolithic ODS

columns. Using 2 m*M* ethylenediamine / 0.1 m*M* Li-DS eluent, the separation of H^+, Na^+, Mg^{2+} and Ca^{2+} was obtained and applied to the analysis of rain water [55]. Though mixtures of the cationic and anionic surfactants described above can be potentially used for simultaneous separations, no such studies have been made to-date.

However, Connolly *et al.* [50] used a different approach for the simultaneous separation of anions and cations, which included the use of two columns. One monolithic column was coated with DDAB (cationic surfactant) and used for anion analysis, whilst a second monolithic column coated with DOSS (anionic surfactant) was used for cation analysis. Both columns were connected in parallel with an eluent of 2.5 m*M* phthalate / 1.5 m*M* ethylenediamine delivered using a flow splitter from a single pump, with simultaneous conductivity detection. Using this system, the separation of up to 8 anions and 5 cations could be achieved in less than 6 minutes.

The synthesis of zwitterionic stationary phases with covalently bonded ion-exchange groups is far more complicated. There are almost no reports in the literature utilising this type of stationary phase for IC. Predominantly, they have been used in biochemical analysis, *e g* a zwitterionic stationary phase for the separation of peptides was prepared by attaching polyethyleneimine to the silica gel with further treatment with hydroxiacetic acid anhydride [58], as shown in Fig. 1-2-3.

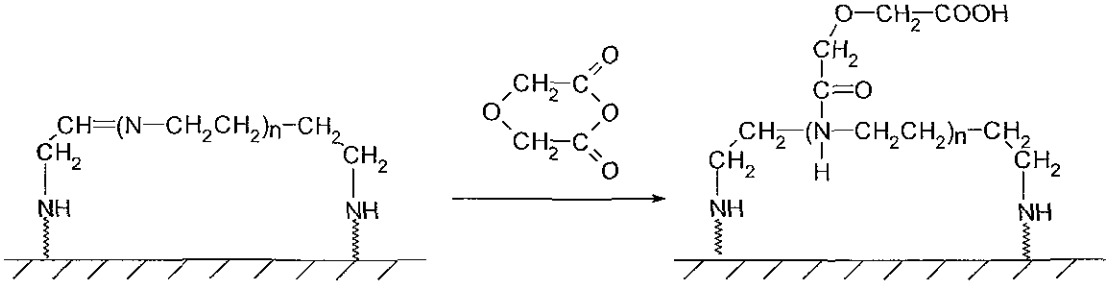


Figure 1-2-3. The synthetic scheme for a multifunctional stationary phase

The authors state that the stationary phase obtained did not exhibit any anion-exchange capacity, because all imine groups were modified. However, by varying the concentration of the hydroxyacetic acid anhydride in the reaction mixture it was possible to obtain zwitterionic stationary phases with different ratios of cation- and anion-exchange capacity.

1 2 5. Zwitterionic stationary phases with attached peptides

Stationary phases with attached peptides are used mostly for chromatographic separation of enantiomers. Ion-exchange properties of these phases have been not fully investigated. Polyampholitic properties of peptide molecules suggest that depending on the pH these stationary phases should exhibit anion- (pH < pI), cation-exchange (pH > pI) or zwitterionic (pH ≈ pI) properties.

Among commercially available peptide-modified stationary phases the most common are bovine serum albumin (BSA, pI = 4.7) modified stationary phases. The ion-exchange properties of silica with attached BSA were investigated by Munaf *et al.* [59]. It was shown that eluent pH had the major influence on anion-exchange properties. At eluent pH < 4 strong anion retention was observed, while increasing pH to 4.6 allowed the separation of a mixture of anions (IO_3^-, Cl^-, NO_2^- and NO_3^-) in less then 10 minutes, using 0.5 m*M* phthalate buffer solution as the eluent. However, under these conditions no retention of cations was observed.

The ion-exchange properties of ODS dynamically coated with BSA has also been investigated [60]. It was found that the optimal pH interval for anion separation was pH 3.2 - 3.6, which can be explained by a lower anion-exchange capacity of the ODS-BSA phase. Additionally, depending on the nature of the initial stationary phase matrix (silica, or ODS), the tertiary structure of attached BSA can be altered, and as a result, its pI.

Kopylov *et al.* [61], investigated cation-exchange properties of silica with covalently bonded sulphonate groups, which were modified with cross-linked BSA globules, and showed that with the increase in number of peptide layers (n) from 0 to 29, the pI for the stationary phase changed from 1.5 to 4.5, which approximates to the pI of the peptide itself. With the increase of n, the retention of cations decreased due to the decrease in the number of sulphonate groups available for ion-exchange, which were screened by the BSA globules. Interestingly, at $n > 15$ an increase in cation retention was observed, which the authors explained by the reduction of ion diffusion into the growing peptide layer.

1.3. Ion-exchangers with two oppositely charged layers of ion-exchange groups on the surface

1.3.1 Centrally localised ion-exchangers with anion-exchange core

A particle of a centrally localised ion-exchanger consists of a core with ion-exchange groups distributed throughout the whole volume and a surface polymer coating, which is inert in relation to the ions being separated. Due to the presence of the inert surface membrane it was possible to obtain stationary phases with relatively big particles and with low ion-exchange capacity, at the same time allowing fast ion transfer to the ion-exchange core. The synthesis of these zwitterionic ion-exchangers was proposed by Dolgonosov [62,63]. The method consisted of treatment of a macro-porous anion-exchange resin with trimethylammonium active functional groups with concentrated sulphuric acid at high temperature. During this reaction the quaternary ammonium group was eliminated from the polymer matrix and the matrix sulphonated with the sulphuric acid. High temperature and viscosity and the low degree of dissociation of sulphuric acid promote the formation of a distinct interface between the aminated core and sulphonated outer surface. Today, centrally localised stationary phases are known under the trade name KanK (Table 1-3-1.) and their ion-exchange properties strongly depend upon treatment conditions, the concentration of sulphonate groups and the depth of surface layer, and can be anion- and cation exchange, or zwitterionic in nature.

Anion retention on centrally localised anion-exchangers depends upon two types of interactions: ion-exclusion on the sulphonated surface and ion-exchange in the aminated core. With the increase of charge of the anion, the repulsion from the surface increases, but at the same time the retention on the positively charged core increases, thus on stationary phases with high anion-exchange capacity the traditional elution order is observed.

Table 1-3-1. Chromatographic properties of KanK centrally localised zwitterionic ion-exchangers [63]

	KanK-ASt	KanK-ASR	KanK-BP
	Anion-exchanger for medium-retained anions	Anion-exchanger for strongly retained anions	Bipolar stationary phase
Anion-/cation-exchange capacity, μequiv/mL	5 – 20 / -	0.05 – 1 / -	100 – 300 / 200 - 300
Efficiency, theor.plates/m	> 12000 (SO_4^{2-})	> 3000 (SO_4^{2-})	> 10000 (Cl^-) > 3000 (NH_4^+)
Test mixtures, elution order and conditions	F^-, Cl^-, NO_2^-, HPO_4^{2-}, Br^-, NO_3^-, SO_4^{2-}; (2.4 m*M* Na_2CO_3 – 3.0 m*M* $NaHCO_3$)	CrO_4^{2-}, WO_4^{2-}, I^-, SCN^-; (3.0 m*M* Na_2CO_3 – 3.0 m*M* $NaHCO_3$)	F^-, CH_3COO^-, $HCOO^-$, Cl^-; (1.0 m*M* Na_2CO_3) Na^+, NH_4^+, K^+; (5 m*M* HNO_3)

1 3.2. Agglomerated ion-exchangers

It is known that beads of cation- and anion-exchange phases attract each other due to electrostatic interactions, which occur between polycation and polyanion particles. This property was used in 1975 by Small *et al.* [1] for the synthesis of agglomerated ion-exchange resins. This method was based on the use of an inert core of sulphonated poly(styrenedivinylbenzene) cation-exchange copolymer, which was coated with a monolayer of electrostatically-bound anion-exchange micro-beads (0.5 - 2.0 μm) of latex. The resultant agglomerated resin acted as an anion-exchanger, but cation-exchange stationary phases can also be produced through attaching cation-exchange particles either to the aminated core or by coating a layer of sulphonated latex particles onto an agglomerated anion-exchange material [1,64]. The most important characteristics of these stationary phases are their chromatographic performance and stability. Efficient separations are possible due to short diffusion paths, leading to excellent mass-transfer characteristics. The high concentration of ion-exchange groups

in a thin layer, formed by the latex micro-particles provides both a high ion-exchange capacity and at the same time, a short diffusion path. The inert and rigid polymer core of the sorbent particles provides the high mechanical and chemical stability. Hydrolytical stability is high in the pH range of 0 – 14, a significant benefit compared to silica-based stationary phases. High cross-link density (up to 55 %) allows the use of these stationary phases with organic solvents, as used in HPLC. Also, the structure of the resin allows the use of high flow rates. These features have led to the wide use of agglomerated ion-exchangers [17,65,65-72]. Today agglomerated ion-exchangers are produced by Dionex and properties of the stationary phase predominantly depend on the structure of the phase.

Despite the fact that agglomerated stationary phases contain both positively and negatively charged ion-exchanged sites, they are predominantly used as monofunctional ion-exchangers, which properties are determined by the nature of ion-exchange groups on the outer layer of micro-beads. However, it is known [69,72] that some agglomerated anion-exchangers possess residual cation-exchange capacity and vice versa. These properties were used for the simultaneous separation of both cationic and anionic forms of chromium (Cr(III) and Cr(VI)) in aqueous samples on the agglomerated anion-exchanger, IonPac AS4A. Interestingly, Cr(III) eluted after Cr(VI), which was evidence of the high residual cation-exchange capacity of the stationary phase [65]. Bruzzoniti *et al.* [72] studied a number of agglomerated ion-exchangers for the simultaneous separation of $HSeO_3^-$ and $TeCl_3^+$, including cation-exchangers IonPac-CS2, CS3, CS10, OmniPac PCX-500 and anion-exchangers IonPac-AS4, AS4A, AS5, AS5A, AS10, AS11. It was shown that $TeCl_3^+$ can be retained on the anion-exchangers IonPac-AS4, AS4A and AS5.

The retention of mono- and dicarboxylic acids on IonPac AS4A-SC was studied by Revercz *et al.* [70] and a change of the retention mechanism with dilution of the eluent was demonstrated. For this type of stationary phases the retention of anions strongly depends on the electrostatic attraction between positively charged amino groups of latex micro-beads on the surface and on the repulsion from sulphonic groups of the particle core. In particular, the impact of ion-exclusion is very strong when diluted eluents are used as the mobile phase. An increase in eluent concentration reduces anion exclusion and the ion-exchange mechanism becomes dominant.

Interesting results were obtained for IonPac CS5 ion-exchangers, which contain both positively and negatively charged latex micro-beads on the surface of the particle

core. This stationary phase was used for simultaneous separation of lanthanides and transition metals [69,73-76]. It was suggested that separation in this case occurs due to cation-exchange, combined with analytes separated as anion complexes by anion-exchange, with the final mechanism being dependent on the concentration of the complexing acid (oxalic, dipicolinic, *etc.*) in the eluent. It was noticed that the elution order of lanthanides due to anion-exchange was opposite of that observed under conditions of cation-exchange and allowed increased possibilities for variation of separation selectivity [69,73].

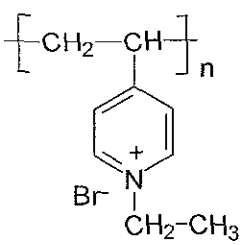
Studies into the synthesis of agglomerated zwitterionic monolithic silica based phases has also been shown [51,77]. Thus, Glenn *et al* [51] used a silica monolith column coated with Dionex AS9-SC latex nano-particles to convert the column into an anion-exchange stationary phase. A complete separation of 7 anions was achieved in approximately 16 minutes using 7.5 m*M* 4-hydroxybenzoic acid. Ikegami *et al.* [77] used a silica capillary column modified with polymethacrylate to obtain the cation- and anion-exchange stationary phase for the separations of anions, nucleobases and nucleotides. The simultaneous separation of eight nucleobases and nucleotides was obtained in 15 minutes using 0.05 *M* $(NH_4)_2HPO_4$, pH 3.0 as the eluent.

1.3.3. Polyelectrolyte-coated stationary phases

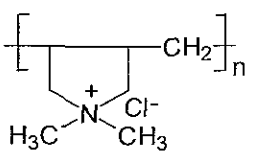
This type of stationary phase can be obtained by modification of the surface of an ion-exchanger with a polymer of opposite charge. The stability of the adsorbed layer is provided by electrostatic interactions between oppositely charged groups. As with stationary phases described earlier, the presence of two oppositely charged layers on the surface of the phase allows faster mass-transfer and as a result, higher ion separation efficiency compared to simple cation- or anion-exchangers.

The first polyelectrolyte-coated stationary phases were synthesised by treatment of 10 μm RP Silasorb C_8 with dodecylbenzenesulphonic acid and further dynamic coating of the resultant weak cation-exchanger (cation-exchange capacity 0.1 - 0.2 mmol/g) with aliphatic ionenes [78,79]. Cation-exchangers modified with aliphatic or aromatic ionenes (polymers with positively charged quaternary ammonium groups) belong to the group of polyelectrolyte stationary phases [78]. Such polyelectrolytes as poly(*N*-ethyl-4-vinylpyridinum bromide) (PEVP), poly(dimethyldiallylammonium) chloride (PDMDAA), poly(hexamethyleneguanidinum)hydrochloride (PHMG) and 2,5-

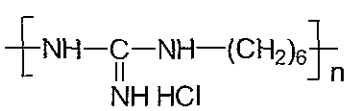
Ionene were investigated as polymers for the outer coating (for structures see Fig.1-3-1.). The structure of the polyelectrolyte and the distribution of the positively charged groups has a major influence on the stationary phase stability. Thus, the stationary phase, modified with PEVP was found to have the most stable coating, while anion-exchange capacity of the phase coated with PHMG decreased by 50 % after 4 L of eluent passed through the column Also, in this work [80], the authors compared aromatic and aliphatic ionenes and showed that aromatic ionenes exhibit similar properties to their aliphatic analogues with shorter chains and high charge density, but were more selective than aliphatic ionenes due to specific π-π interactions.



PEVP



PDMDAA



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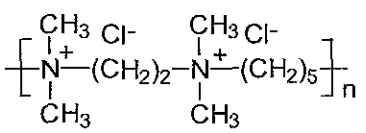


2-5 Ionene

Figure 1-3-1. The structures of polyelectrolytes

An interesting dependence of anion-exchange selectivity on the ionene structure was presented by Pirogov *et al.* [81]. Aliphatic ionenes of x,y-Ionene type (2-4, 3-4, 2-8, 3-8, 3-6, 4-6, 6-8 and 6-10) were used as an example. It was shown that stationary phases modified with ionenes with high a distribution density of quaternary ammonium groups (2-4, 3-4) exhibit strong affinity for sulphate, whilst their selectivity to easily polarised ions (I^-, SCN^-, ClO_4^-) was poor. For stationary phases modified with ionenes, where the numbers of methylene groups between nitrogen atoms are $x < 5$ and $y > 5$, sulphate retention was still high. At the same time, selectivity for I^-, SCN^- and ClO_4^- increased. Relatively low sulphate retention, which elutes before thiocyanate, was observed for 6-8 and 6-10 ionenes. The authors attribute the increase in retention for SCN^- and ClO_4^- to the increase of hydrophobic interactions between these anions and the methylene groups of the ionenes. However, experimental data also showed a

decrease in retention for sulphate, which can be related to the repulsion from the sulphonated matrix of the stationary phase.

Cation-exchangers modified with ionenes were also used for the separation of anions. The authors [78] suggested fast and efficient methods for the simultaneous separation of weakly and strongly retained anions, and the following elution order was observed with a potassium hydrogen phthalate eluent:

$$IO_3^- < Cl^- < H_2PO_4^- < NO_3^- < SO_4^{2-} < I^- < SCN^- < ClO_4^-.$$

1.3.4. Polysaccharide-coated stationary phases

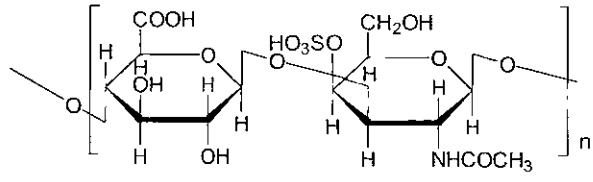
Zwitterionic stationary phases can be obtained by the dynamic modification of an anion-exchanger surface with polysaccharides containing carboxyl and/or sulphonate groups. The synthetic method is similar to that for obtaining polyelectrolyte stationary phases. Polymer molecules are attached to the surface of the matrix due to electrostatic interactions between oppositely charged groups.

Modification of an anion-exchanger, namely silica TSK-gel IC-Anion-SW, with an anion-exchange capacity of 0 4 mequiv/g, was carried out using sodium chrondroitin sulphates [82,83], heparin [83-86] and dextran sulphates [87,88] (for structures see Fig. 1-3-2).

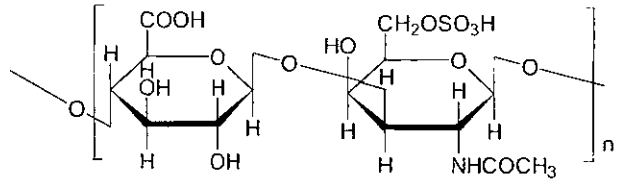
Along with polysaccharides, poly(styrenesulphonate) and *N*-acetylglucosamine-6-sulphate, which is the building block for the studied polysaccharides, were also used for the modification of anion-exchange stationary phases. However, stationary phases modified with these reagents were found to be unsuitable for chromatographic separations [83].

It was shown that modification of an anion-exchanger surface with polysaccharides reduces anion retention due to the decrease of anion-exchange capacity of the stationary phase and repulsion of anions from the outer negatively charged layer. This effect was the strongest for stationary phases coated with heparin. Also, a very unusual effect showing the decrease in retention of analyte anions with a decrease in eluent concentration was observed. This was explained by the fact that on the surface of the stationary phase, analyte anions experience both attraction to positively charged groups of the anion-exchanger and repulsion from negatively charged functional groups of the polysaccharide. So the increase in the ionic strength of the eluent decreased the repulsion of anions from the negatively charged groups, whilst simultaneously

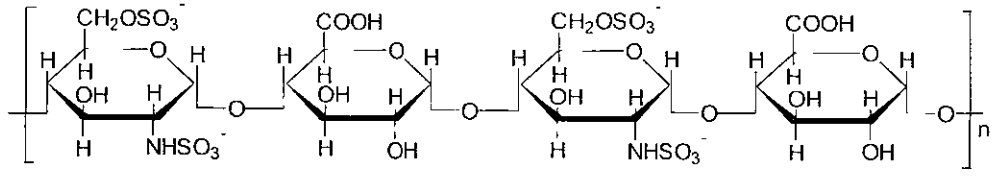
providing increased eluting ions in the normal ion-exchange process. The total effect results in a reduction in the retention time for analyte anions with a dilution of the eluent. This facilitated the use of diluted eluents, which resulted in more sensitive conductivity detection.



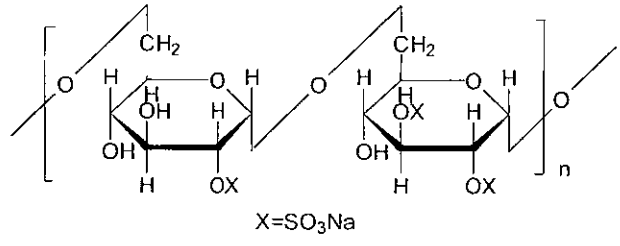
Chrondoitin sulphate A



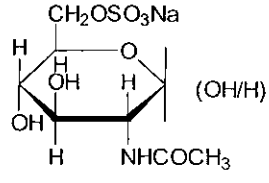
Chrondoitin sulphate C



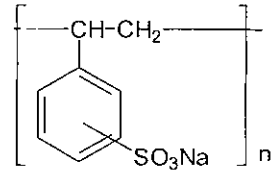
Heparin



Dextran sulphate



N-acetylglucosamine-6-sulphate



Poly(styrenesulphonate)

Figure 1-3-2 Structures of polysaccharide modifiers

Interestingly, anion retention was also shown to strongly depend upon the nature of the eluent cation. Thus on the heparin-modified stationary phase, it was shown that the retention of nitrate increases with each of the following eluents in the order shown:

$$LiCl < NaCl < KCl < RbCl < CsCl.$$

Simultaneous separation of cations and anions, presented in Fig. 1-3-3, was obtained on a TSKgel IC-Anion SW column modified with heparin, with a 1 m*M* $CuSO_4$ eluent. Low retention of cations compared to anions is evidence of the fact that despite the polysaccharide coating, there were still enough amino groups available for anion-exchange.

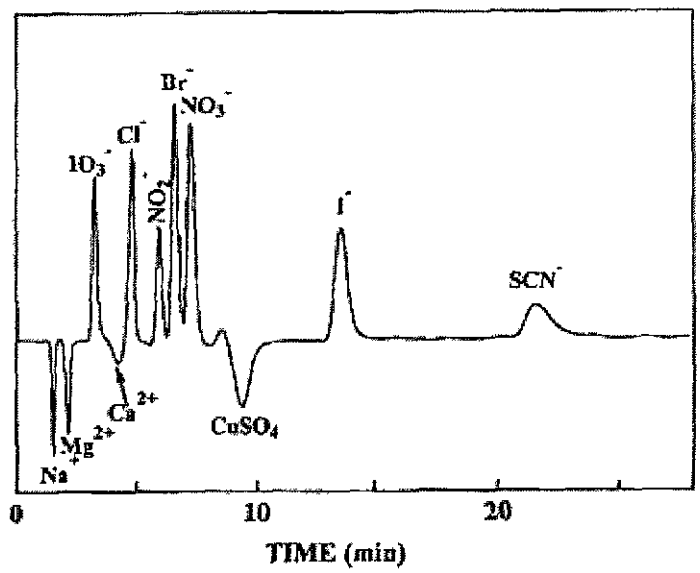


Figure 1-3-3 The chromatogram of model mixture of cations and anions Column: TSKgel IC-Anion SW (100 x 0.32 mm I.D.), modified with heparin Eluent: 1mM $CuSO_4$, UV detection at 200 nm Reprinted from [85]

The ratio of the cation- and anion-exchange capacities of this type of zwitterionic stationary phases depends upon the characteristics of the initial anion-exchanger and on the molecular weight of the polysaccharide itself. Thus, for the heparin coated stationary phase, (weight-average molecular mass ~ 2200) the data shown in Table 1-3-2 was recorded [89]. It can be seen from Table 1-3-2 that one of the main factors affecting capacities is the pore diameter. When the size of the pore is smaller than the size of dextran sulphate, anion-exchange sites existing in the pore remain unmodified. If pores are large, it is easier for the polysaccharide molecules to get into the pores. Since it is also expected that not all of the sulphate groups of the modifier interact with the anion-exchange sites of the anion-exchanger, a portion of

sulphate groups on the modifier remain free, and act as cation-exchange sites. Since an anion-exchanger modified with polysaccharides therefore possesses both anion- and cation-exchange sites, it again facilitates the possibility of the simultaneous separation of cations and anions. It is clear that polysaccharide molecular weight has the same effect. The smaller molecules result in more complete coating and anion-exchange capacity is smaller.

Table 1-3-2 Ion-exchange capacities of stationary phases before and after modification with heparin [89]

Stationary phase	Before modification				After modification	
	S, m^2/g	d_{pores}, nm	V_{pores} mL/g	Anion-exchange capacity, μequiv/mL	Anion-exchange capacity, μequiv/mL	Cation-exchange capacity, μequiv/mL
1	181	14	0.79	160	2.1	32
2	238	16	1.06	110	3.9	21
3	197	23	1.27	150	-	30
4	197	36	1.10	66	-	31

1.3.5. Poly(amino acid) functionalised stationary phases

A significant drawback of stationary phases dynamically coated with polyelectrolytes and polysaccharides is the noticeable bleeding of the modifier from the column [84]. A possible solution to this problem involves the covalent attachment of an anionic polymer to a weak anion-exchanger. This approach was used for preparation of poly(aspartic acid) bonded silica [90]. Two successive treatments with aminopropyltriethoxysilane and polysuccinimide resulted in the ion-exchanger schematically shown in Fig. 1-3-4.

Due to the zwitterionic properties of amino acids, such poly(aspartic acid) stationary phases, under trade name PolyCAT A (PolyLC), were investigated for the simultaneous separation of cations and anions [91-93]. It was found that this stationary phase exhibited selectivity to both cations and anions. The effect of eluent pH and concentration, and the nature of the eluent cation on separation selectivity were evaluated. It was shown that these parameters had a strong effect on the retention of both sets of ions. The following elution order with a $LiSO_4$ eluent was observed [91]:

$$Cl^- < Br^- < H_2PO_4^- < I^- < IO_3^- < NO_3^- < SCN^- < Li^+ < Na^+ < NH_4^+ < K^+ < Mg^{2+} < Ca^{2+}.$$

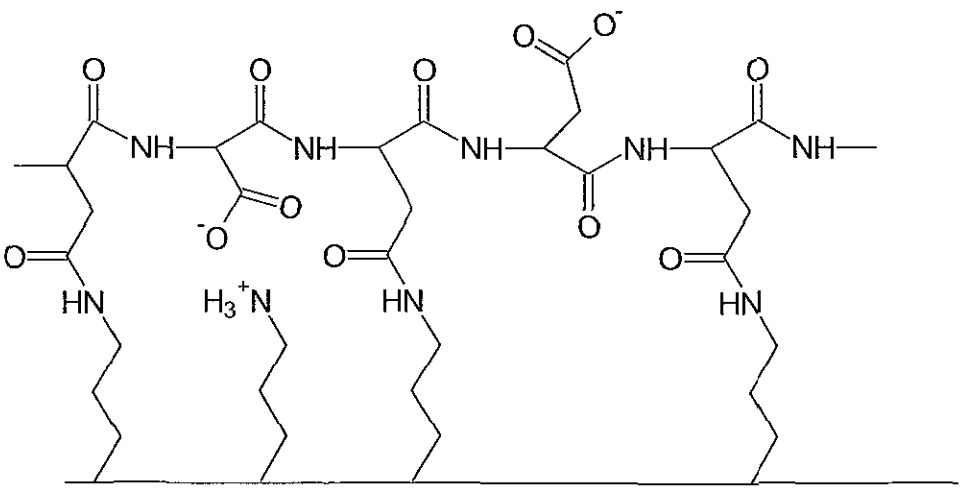


Figure 1-3-4 Structure of the bonded layer of poly(aspartic acid) – silica

It was also found that the ion-exchange selectivity depended upon the silica porosity. Thus the elution order for anions changed from,

$$Cl^- < Br^- < ClO_3^- < BrO_3^- < I^- < IO_3^- < NO_3^- < SCN^- < ClO_4^-$$

for 30 nm pore PolyCAT A to,

$$I^- < Cl^- < ClO_3^- < ClO_4^- < BrO_3^- < Cl^- < SCN^- < IO_3^- < NO_3^-$$

for 6 nm pore PolyCAT A [92]. Authors explained this phenomenon by the inability of the polymer coating to permeate narrow pores and as a result, a network of poly(aspartic acid) blocked the opening of the pores. This would account for an ion-sieving effect; whereby electrostatic repulsion of anions by the carboxylic acid groups would regulate their access to the amino groups within the pores, resulting in the observed reversal in retention order.

1.4. Zwitterionic ion-exchangers with covalently attached or physically adsorbed zwitterionic molecules

1.4.1. Stationary phases with covalently bonded zwitterionic molecules

This group of stationary phases includes zwitterionic ion-exchangers with covalently bonded amino- and either sulpho- or carboxyl groups, which belong to one molecule. These ion-exchangers can be synthesised by bonding the organic moiety to the silica surface, with further reactions to obtain necessary groups on the surface, or by covalent bonding of bipolar molecules. Both synthetic approaches were used [94] for

the synthesis of the stationary phases for the separation of aromatic acids, bases and ampholites. The zwitterionic stationary phase was obtained by sulphonation of phenylaminoethyl groups, attached to the surface of the silica, as shown in Fig. 1-4-1. Depending on synthetic conditions, it was possible to obtain stationary phases with different ratios of cation- and anion-exchange groups on the surface. However, total sulphonation of all phenylaminoethyl groups was not possible, so on the surface of the ion-exchanger, along with zwitterionic groups, anion-exchange groups remained present. This can cause confusion in the investigation into the retention mechanism on these type of stationary phases.

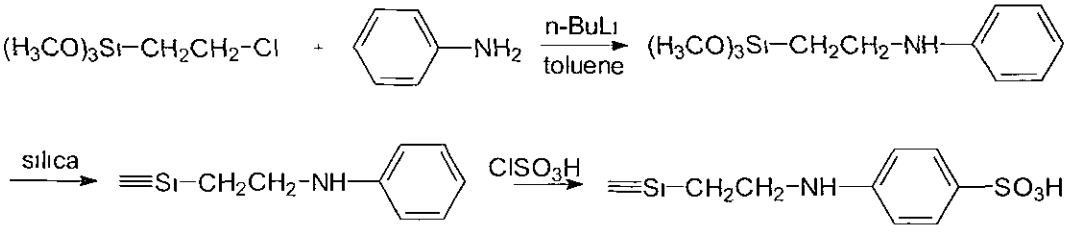


Figure 1-4-1. The synthetic scheme for ion-exchange stationary phase formed by surface assembling method

However, this can be avoided if pre-synthesised zwitterionic molecules are attached to the surface of the stationary phase as shown in Fig 1-4-2. Based on the retention dependences on pH for aromatic acids, bases and ampholytes, authors suggest that retention on both stationary phases occurs mainly due to anion-exchange. The retention of organic cations is weak and occurs due to hydrophobic π-π interactions.

Hartwick *et al.* [95] synthesised silica-based stationary phases with bonded bipolar molecules, which contain both quaternary amino group and sulphonate group as shown in Fig. 1-4-2. To obtain a constant positive charge on the nitrogen atom, it had to be methylated. This process, especially if the reaction takes place on the surface of the stationary phase, is labored due to the presence of bulky groups. So it is most likely that besides quaternary amino groups being present, there are also secondary and tertiary amino groups produced. These zwitterionic ion-exchangers were used as universal stationary phases for RP HPLC, cation-, anion- and zwitterion-exchange chromatography modes Unfortunately, selectivity of these stationary phases to inorganic ions was not investigated. Peptides, amino acids and pharmaceutical substances were used as analytes

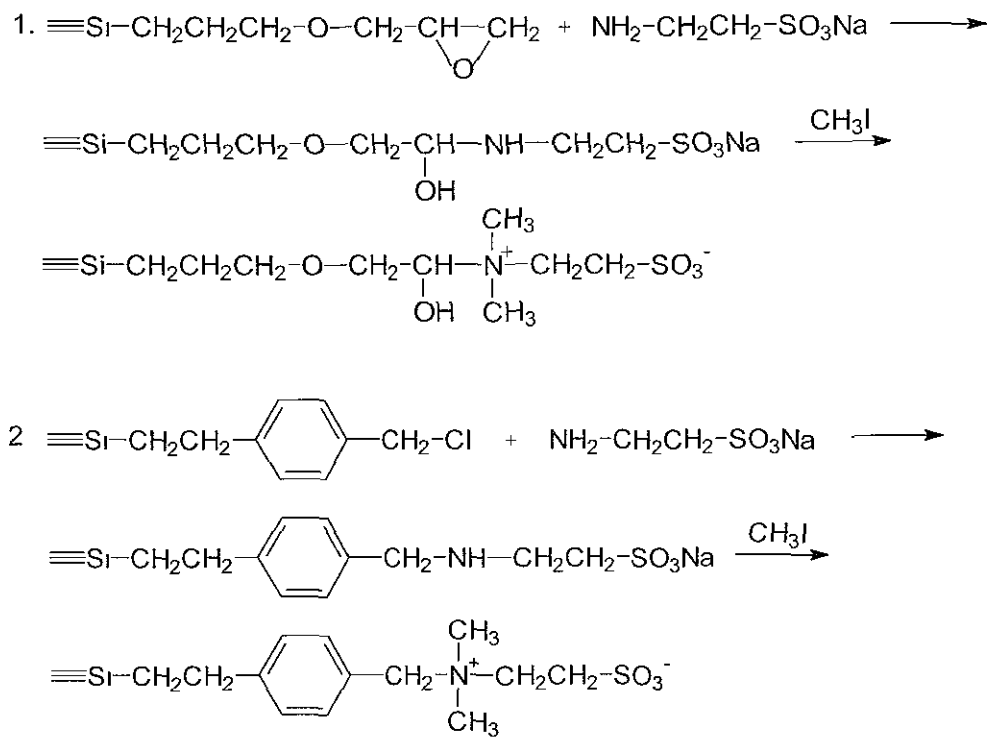


Figure 1-4-2 The synthesis scheme of zwitterionic ion-exchanger with bonded bipolar molecules

Irgum *et al* [96] proposed a new synthetic method for zwitterionic stationary phases. Modification of an activated cross-linked 2-hydroxyethyl methacrylate polymer matrix with 2-(dimethylamino)ethanesulphonic acid (DMAES) resulted in a zwitterionic ion-exchanger, with an ion-exchange centre according to the scheme shown in Fig 1-4-3.

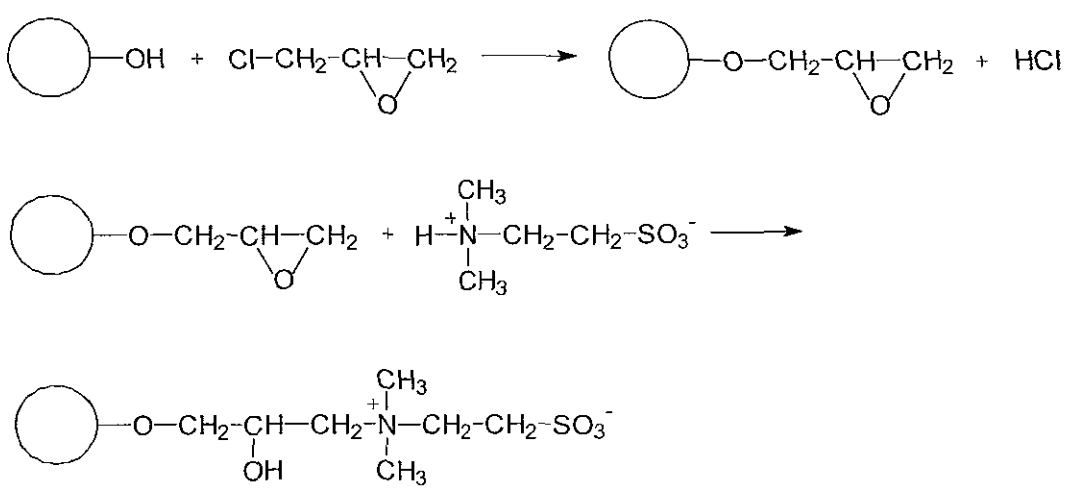


Figure 1-4-3. Synthetic scheme for a zwitterionic stationary phase based on cross-linked 2-hydroxyethyl methacrylate polymer beads modified with DMAES [96]

The elution order of anions on the obtained stationary phase, using a 2.5 m*M* $NaClO_4$ as eluent was as follows:

$$SO_4^{2-} < F^- < Cl^- < NO_2^- < Br^- < NO_3^- < I^-.$$

Weak retention of sulphate due to the repulsion from the external cation-exchange groups of the stationary phase was observed. These zwitterionic ion-exchangers show low selectivity to cations, and separation is only possible for groups of cations of different charges 2 m*M* $HClO_4$ was used as an eluent for the simultaneous separation of cations and anions (SO_4^{2-}, Cl^-, Br^-, Na^+ and K^+, NO_3^-, Ca^{2+}, I^-) It was shown that an increase in $HClO_4$ concentration, up to 10 m*M* lead to a reduction in the retention of anions, but at the same time the capacity factor values for cations increased. The authors explain this phenomenon by an increase in the screening of positively charged groups by perchlorate anions. This leads to a decrease in the repulsion of cations from positively charged groups within the zwitterionic ion-exchanger. An attempt to separate inorganic cations and anions using pure water as eluent was made by Irgum *et al.* [96]. Na^+ and Ca^{2+} cations were separated, however, on the chromatograms shown negative peaks were observed before and after positive cation peaks.

1 4.2. Silica-based stationary phases with attached amino acids

Another approach to obtain zwitterionic ion-exchangers is via the covalent attachment of α-amino acids onto the surface of a matrix such as silica. Compared to all the stationary phases described earlier, the main advantage of amino acid modified stationary phases is the possibility of selectivity variation due to controllable cation- and anion-exchange capacities through changes in pH. Another advantage of these phases is the possibility of retention due to the complexation of the separation of transition and rare-earth metal ions [97]. Although silica-based ion-exchangers with attached amino acids have been successfully used for the preconcentration of transition metals [98], affinity chromatography [99], ligand-exchange [100] and standard IC [10], the full range of ion-exchange properties of stationary phases modified with α-amino acids have been only studied relatively recently [101-111]. The attachment of α-amino acids is carried out according to the following reaction scheme [103], which is shown in Fig. 1-4-4, where R_1 and R_2 depend on the structure of the attached amino acid.

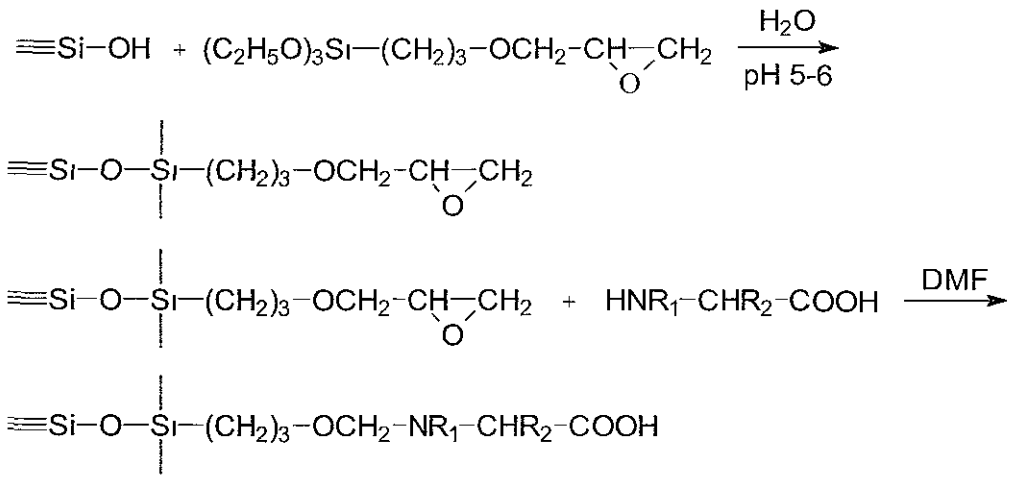


Figure 1-4-4. Reaction scheme for synthesis of amino acid-bonded silica

The influence of the amino acid structure was investigated earlier by Nesterenko *et al.* [101-103]. It was shown that stationary phases modified with mono-amino di-carboxylic (aspartic, glutamic acids), mono-amino mono-carboxylic (valine, tyrosine) and di-amino mono-carboxylic (lysine, arginine) acids, predominantly exhibited cation-exchange properties. For lysine and arginine this was unexpected, as pI values for these amino acids are 9.82 and 10.76, respectively [112]. However, due to the activity of matrix silanol groups, and their ability to form hydrogen bonds with amino group nitrogen [98], such amino groups are not available for anion-exchange. As a result the structures, such as that presented in Fig. 1-4-5 for lysine attached to the silica surface, can occur.

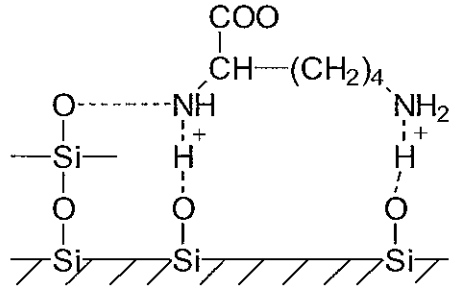


Figure 1-4-5. Hydrogen bonds between silanol groups on the surface and amino groups of the amino acid

Alternatively, silica with attached arginine groups exhibited weak anion-exchange properties. Anions, such as Cl^-, ClO_4^- and SO_4^{2-} (as in elution order) were partly separated using very diluted eluents (~ 10^{-4} *M*). Unlike lysine, arginine has three basic nitrogen atoms in very close proximity, so the residual silanol groups on the

surface are not in a high enough concentration to form a hydrogen bond with each of these atoms [106].

As amino acids on the surface can exist in several forms, depending upon the pH, both cation- and anion-exchange properties can be observed and simultaneous separations of cations and anions are possible. In work conducted by Nesterenko *et al.* [104], the simultaneous separation of cations and anions was shown on proline and hydroxyproline coated silica. Depending on the eluent pH, the forms of these amino acids, shown in Fig. 1-4-6, were present. The most appropriate pH range for this application was pH 4 to 6, which corresponds with the zwitterionic form of the attached amino acid. The elution order for hydroxyproline coated silica with a 5 m*M* lithium benzoate eluent, pH 5.2, was:

$$SO_4^{2-} < H_2PO_4^{2-} < Cl^- < NO_3^- < ClO_4^- < \text{system peak} < Li^+ < Na^+ < NH_4^+ < K^+ < Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+} < CH_3COO^- < Zn^{2+}$$

The observed selectivity and efficiency of the method was found to be high enough for the simultaneous separation of 7 cations and anions [104].

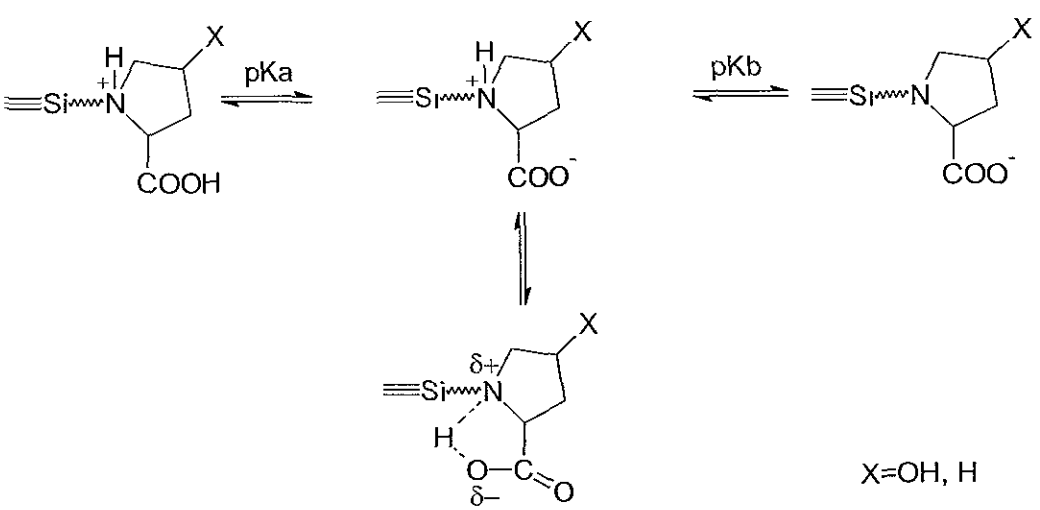


Figure 1-4-6. Scheme of equilibrium ionic forms for amino acids, example of proline and hydroxyproline

Yu *et al.* [113] showed the simultaneous separation of organic acids and bases using a silica stationary phase with attached glutamic acid molecules. The synthesis of the glutamic acid phase was achieved according to the scheme shown in Fig. 1-4-7.

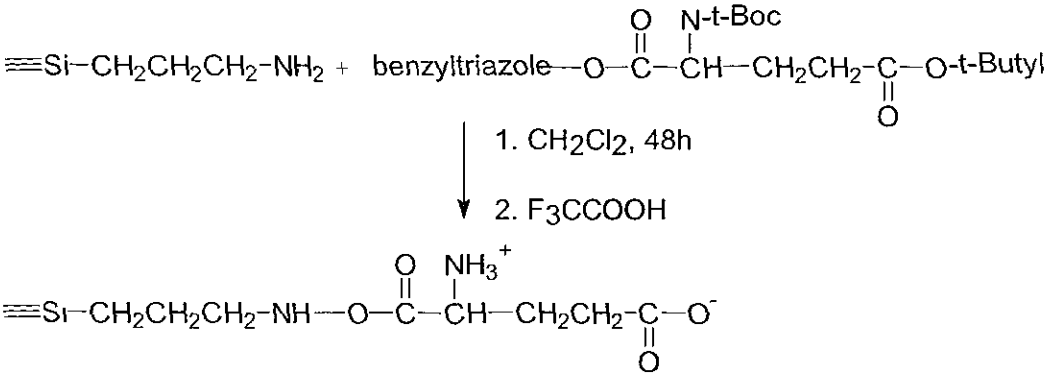


Figure 1-4-7. The synthetic scheme for glutamic acid bonded silica

pK_a values for carboxyl and amino groups of glutamic acid are 3.0 and 9.7, respectively. Varying eluent pH makes it possible to find such conditions, where retention occurs predominantly due to cation-exchange (pH ~ 7) or anion-exchange (pH ≤ 4). At the pH range between 4 and 7, simultaneous separation of anions and cations is possible.

The formation of a triple layer structure of positively charged amino groups, between the negatively charged silica silanols and the carboxylic cation-exchange groups, due to interactions between basic amino groups and residual acidic silanol groups, should provide good mass-transfer characteristics and a short diffusion path and hence high chromatographic efficiency of these type of ion-exchangers [101,102,106]. This structure was also shown to reduce the retention of alkaline-earth metal cations allowing the separation of alkali and alkaline-earth metal cations simultaneously in only 10 minutes, a task impossible with traditional sulphonic or carboxylic cation-exchangers, for which the affinity to single- and multi-charged cations differs significantly.

It is interesting to compare selectivities of zwitterionic ion-exchangers with attached proline and arginine groups. Proline has two oppositely charged ion-exchange groups in close proximity. The retention of ions depends upon the sum of attraction to oppositely charged groups and repulsion from the groups with the same charge. This results in a relative reduction of retention time for sulphate on proline modified stationary phases. At the same time, arginine has two anion-exchange centres: an amino group in α-position and a guanidine group. It has been shown that retention of anions is dependent predominantly upon the latter.

The weaker anion retention on stationary phases with attached hydroxyproline compared to traditional anion-exchangers was utilised for rapid separation of a mixture

of inorganic anions (NO_2^-, IO_3^-, $H_2PO_4^{2-}$, Cl^-, Br^-, NO_3^-, I^-, ClO_4^-, SCN^-). At an optimum pH of 3.13, it was possible to separate nine anions in less than 10 minutes [103]. Relatively low retention, compared to mono-functional ion-exchangers, of bulky anions like I^-, ClO_4^- and SCN^- can be explained by their repulsion from dissociated carboxyl groups of the attached amino acid. The increase in size of the ion results in an increase in repulsion. It was also observed that retention of ampholyte organic molecules, such as amino acids and nucleosides, occurs due to highly selective "quadrupole" interactions between the zwitterionic molecule on the surface and the analytes [95,113].

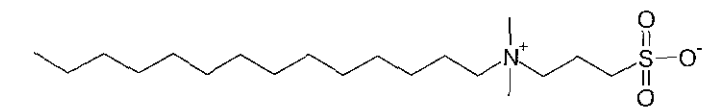
1.4.3 Stationary phases dynamically coated with zwitterionic molecules

One of the most simple ways to obtain bipolar ion-exchangers is dynamic modification of RP sorbents with zwitterionic reagents. The most common sorbent is ODS, which is modified with a hydrophobic zwitterionic surfactant in which positively and negatively charged functional groups are separated by methylene chains of differing length.

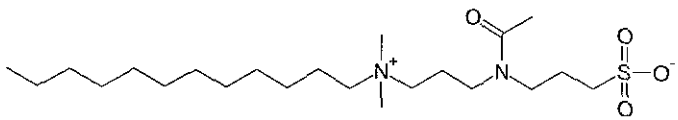
A number of common zwitterionic surfactants are shown in Fig. 1-4-8 and include 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS) [114,115], 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonate (CHPSO) [116], *n*-tetradecyl-*N,N*-dimethyl-3-ammonio-1-propanesulphonate (Zwittergent 3-14) [117-120], *N*-(3-acetyl-3sulphopropyl)aminopropyl-*N,N*-dimethyldodecanaminium hydroxide (ammonium sulphobetaine-3) [121], sodium taurodeoxycholate and sodium taurocholate [122], and (dodecyldimethylammonio)acetic acid (DDMAA) [123,124]. Stationary phases modified with the above surfactants have been used for the separation of organic and inorganic anions using water or diluted eluents with non-suppressed or suppressed conductivity detection [114,115,118,119,125-128].

Due to the presence of a bulky hydrophobic region, the surfactant molecules are strongly retained on the surface of ODS, resulting in stable ion-exchangers. In these zwitterionic molecules, opposite charges are located in close proximity. This means that analyte ions experience not only attraction to oppositely charged groups, but also repulsion from groups with the same charge. The resulting interactions are relatively weak, allowing the use of diluted eluents or even pure water as the mobile phase.

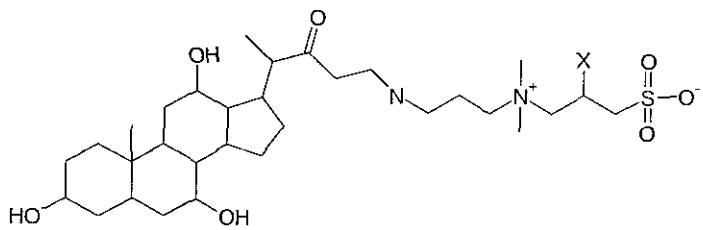
The combined effect of the simultaneous electrostatic forces is dependent on the charge and radius of the ion. This means that the simultaneous electrostatic attraction and repulsion interactions between the same and opposite charges can be used for the separation of differently charged ions. The chromatographic method based on the simultaneous attraction and repulsion of analyte ions by ion-exchange sites on the zwitterionic stationary phase, using pure water as mobile phase, was termed electrostatic ion chromatography (EIC) [129]. To obtain such zwitterionic ion-exchangers, either individual surfactants [116,129,130] or their mixtures [43,52,131,132] have been used.



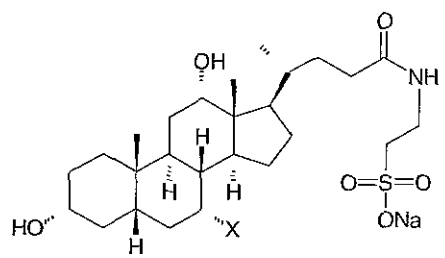
Zwittergent 3-14



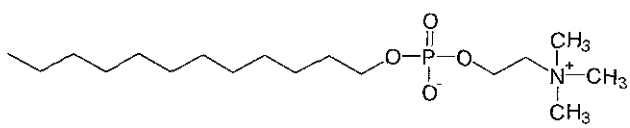
Ammonium Sulphobetaine-3



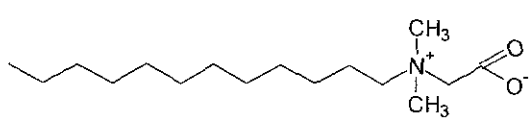
CHAPS (X=H), CHAPSO (X=OH)



Taurodeoxycholic acid (X=H); Taurocholic acid (X=OH)



N-dodecylphosphocholine



(Dodecyldimethylamino)acetic acid (DDMAA)

Figure 1-4-8. Structures of zwitterionic surfactants used for preparation of coated zwitterionic ion-exchangers

The retention of ions on ODS dynamically modified with zwitterionic surfactants depends on the nature of ion-exchange groups of the surfactant and on its

surface concentration. The surface concentration of the surfactant depends on the size of the bipolar group (*e g.* the length of the methylene chain between opposite charges) and on the size and hydrophobicity of the non-polar part of the molecule. For ODS, modified using the same conditions with different sulphobetaine type surfactants, it was shown that the surface concentration increased in the following order: CHAPS < Zwittergent-3-12 < Zwittergent-3-14 [133]. A bulky hydrophobic region increases the affinity of the surfactant, such as CHAPS to the RP sorbent, however, it also simultaneously restricts the number of the molecules adsorbed on the surface. It was shown that with an increase in the surface concentration of surfactants, retention for the majority of anions increased and the peak resolution improved. The elution order of anions on ODS dynamically coated with Zwittergent-3-12 with water as the eluent was [133]:

$$F^- < IO_3^- < SO_4^{2-} < CH_3COO^- < S_2O_3^{2-} < Cl^- < NO_2^- < Br^- < NO_3^- < ClO_3^- < I^- < SCN^- < ClO_4^-.$$

It was found that this type of stationary phase did not show selectivity for cations of the same charge, as they coeluted and separation of only cations of different charges was possible [116]. At the same time these stationary phases exhibited high selectivity to anions and the separation of 8 common anions can be seen in Fig. 1-4-9. The low retention of sulphate was caused by a strong repulsion from the cation-exchange groups of the surfactant.

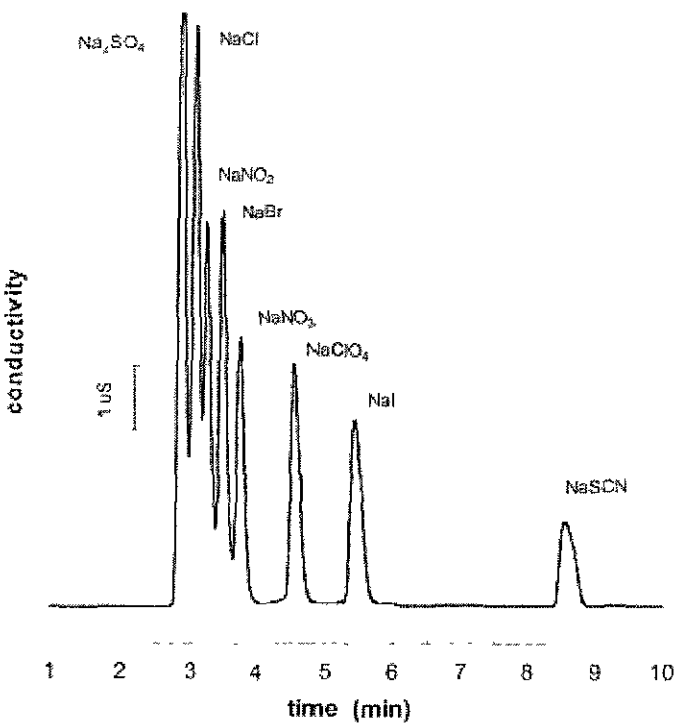


Figure 1-4-9. Chromatogram of a mixture of sodium salts of eight anions. Column L-Column ODS 250 x 4.6 mm I.D. coated with CHAPS – Zwittergent 3-14; eluent pure water, conductivity detection. Reprinted from [134]

There is a large volume of work published on the application and investigation of properties of sulphobetaine type surfactants. Thus, using ODS coated with 3-(*N,N*-dimethylmyristylammonio)propanesulphonate and pure water as an eluent, Hu *et al.* [135] determined bromide, nitrate and iodide in sea water at sub-ppb levels. A zwittergent-3-14 coated phase was also used for the anion analysis of similar saline samples [120]. It is worth noting that for this separation Zwittergent-3-14 was also used as the eluent. An attempt to separate cations using a Zwittergent-3-14 phase and water as the eluent [136] was also made, but it was not possible to separate cations of the same charge.

When a surfactant with an outer anion-exchange group and inner cation-exchange group was used, it was observed that cations were more strongly retained than anions, as was shown for a *N*-dodecylphosphocholine zwitterionic stationary phase [137]. The elution order for alkaline-earth and transition metal cations corresponded with known complex stability constants of these ions with the phosphate group:

$$K^+ < Ba^{2+} < Mg^{2+} < Ca^{2+} < Mn^{2+} < Cu^{2+} < Zn^{2+}.$$

Recently, the use of carboxybetaine type surfactants for coating ODS stationary phases was investigated. These surfactants contain an inner anion-exchange quaternary amino group and terminal cation-exchange carboxylic acid group. The first carboxybetaine type surfactant used was Mitsubishu Reagent ($C_8F_{17}SO_2NHC_3H_6N^+(CH_3)_2$-$C_2H_4$-$COO^-$), which Hu *et al.* [138] used for the separation of anions with 1 m*M* H_2SO_4 as the eluent. The retention order obtained for this stationary phase was:

$$NO_2^- < H_2PO_4^- < Cl^- < Br^- < NO_3^- < ClO_3^- < I^-.$$

O'Riordain *et al* [123,124] reported interesting selectivity on both packed and monolithic columns modified with (dodecyldimethylammonio)acetic acid. It was shown that the retention of anions could be varied with pH, and it was proposed that at low pH due to protonation, the repulsion between analyte anions and the carboxylic acid group decreases and the attraction to the positively charged quaternary ammonium group increases, thus increasing anion retention. Separation of 5 anions was obtained using a 10 m*M* phosphate buffer in 10 m*M* KCl and the retention order was:

$$NO_2^- < Br^- < NO_3^- < I^- < SCN^-.$$

Recently, several studies have been carried out using DDMAU [139,140]. O'Riordain *et al.* [139] used a RP monolithic capillary column for the separation of 9 anions using a 0.5 m*M* phthalate (pH 4.0) eluent with contactless conductivity detection (C^4D). The retention order was:

$$BrO_3^- < NO_2^- < Br^- < NO_3^- < I^- < SO_4^{2-} < S_2O_3^{2-} < SCN^- < ClO_4^-.$$

At the same time, Barron *et al.* [140] showed that DDMAU-modified stationary phases exhibit very strong selectivity to perchlorate ion and applied this property for the on-line preconcentration of perchlorate, and its subsequent determination in rainwater samples.

The retention mechanism for ions on zwitterionic phases such as those described above, when using water only as the mobile phase, differs from that for monofunctional stationary phases where an electrolyte solution is used as eluent. Deionised water contains anions and cations, which are formed by water autoprotolysis and as a result of the dissolution of gases, particularly CO_2. However, their concentration is not enough to maintain electroneutrality of the eluted solution, so ions are eluted as ion pairs [116]. Therefore, a hypothetical chromatogram of a mixture of cations $C_1 \ldots C_n$ and anions $A_1 \ldots A_m$ will contain n×m peaks, based upon all possible ion pairs:

$$\begin{bmatrix} C_1 \\ \vdots \\ C_n \end{bmatrix} \times \begin{bmatrix} A_1 \cdots A_m \end{bmatrix} = \begin{bmatrix} C_1A_1 \cdots C_1A_m \\ \vdots \quad \cdots \quad \vdots \\ C_nA_1 \cdots C_nA_m \end{bmatrix}.$$

However, some of these ion-pair-like species may not be observed in the separation because of their low probability of formation. The ability of cations and anions to form ion pairs depends on their molal energies ΔG, which are dependent on the charge and the hydrated radius of the ion. The molal energy ΔG can be calculated by Born equation:

$$-\Delta G = \frac{(1 - \frac{1}{\varepsilon})e^2}{2r} \qquad \text{(eq. 1-4-1)}$$

where e is the charge, r – radius immersed in the medium, ε - dielectric constant.

An example separation is shown in Fig. 1-4-10. The chromatogram of the mixture of ions Na^+, Ba^{2+}, Cl^-, SCN^- shows four peaks, corresponding to the following ion pairs: [Na^+ - Cl^-], [Ba^{2+} - $2Cl^-$], [Na^+ - SCN^-] and [Ba^{2+} - $2SCN^-$]. To prove the supposition of adsorption of ion pairs, ICP-AES detection was used. It was shown that

all peaks on the chromatogram, where conductivity detection was used, correspond to the appropriate ion pair.

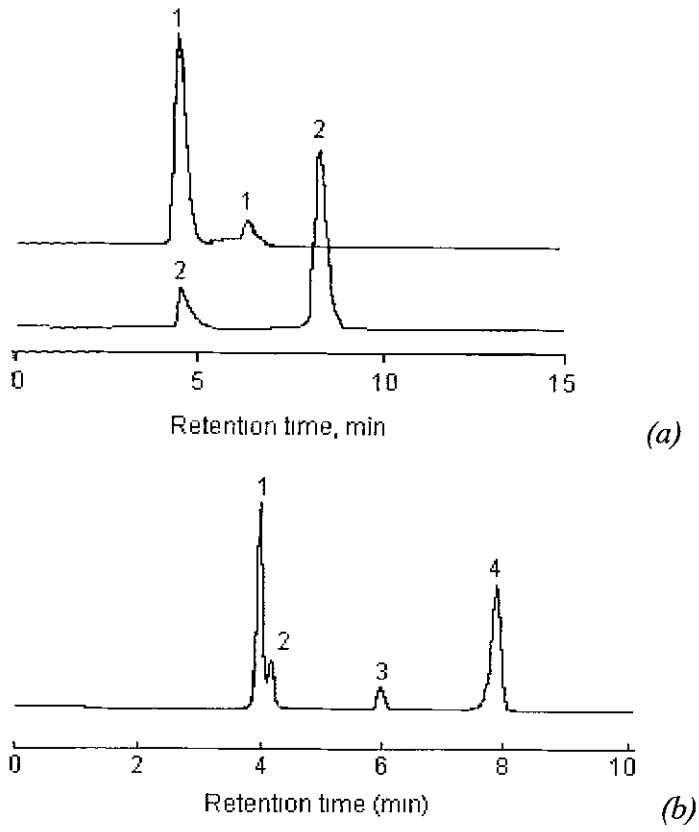


Figure 1-4-10. Chromatogram of an aqueous solution of NaSCN and $BaCl_2$. Column L-Column ODS, 250 x 4.6 mm I.D, coated with CHAPSO; eluent: pure water, detection: (a) ICP-AES, (b) UV absorption at 230 nm; (a) 1 - Na^+; 2 - Ba^{2+},(b) 1 – [Na^+ - Cl^-]; 2 – [Ba^{2+} - $2Cl^-$], 3 – [Na^+ - SCN^-]; 4 – [Ba^{2+} - $2SCN^-$]. Reprinted from [116]

The areas of the first and last peaks on both chromatograms are several times larger than areas of the remaining peaks, which proves the predominant formation of the corresponding ion pairs [116,141]. The retention of these ion pairs depends on the nature of ions forming the pair, and especially on the nature of the anions [142]. The retention times for ion pairs, obtained on ODS coated with Zwittergent-3-14, with water as the eluent are shown in Table 1-4-1 [143]. It is clear that stationary phase selectivity was higher for anions than for cations, and only the separation of cations with different charges was possible.

Table 1-4-1. Retention times for ion pairs in EIC mode

	Cl^-	NO_2^-	Br^-	NO_3^-
Na^+	4.38	4.83	5.96	6.91
K^+	4.40	4.86	6.03	6.97
Mg^{2+}	4.92	5.81	8.21	10.62
Ca^{2+}	4.96	5.86	8.32	10.82

This ability of ions to elute as ion pairs in EIC mode was used for the determination of Br^- and I^- in sea water [117,118]. It is hard to determine Br^- in a high abundance of Cl^-, however the addition of $MgCl_2$ (> 50 m*M*) to the sample increases the retention of Br^- due to formation of the ion pair [Mg^{2+} - $2Br^-$] and as a result, separation selectivity for Cl^- and Br^- increases.

An unusual approach for the detection of anions in EIC mode was used by Umemura *et al* [136]. The chromatographic system consisted of two coupled columns: the first was a separation ODS column, coated with Zwittergent-3-14, and the second was cation-exchange column in Na^+- or Mg^{2+}- form. As a result all anions eluting from the second column had Na^+ or Mg^{2+} as a counter-cation in the ion pair. So it became possible to determine concentration of anions by determining the cation using sensitive methods such as atomic adsorption spectrometry, atomic emission spectrometry with inductively-coupled plasma or mass-spectrometry. The use of water as an eluent increased the sensitivity of the detection method.

Another approach for the elimination of multiple peaks in EIC was suggested by Hasebe *et al.* [144]. This method used two columns, one cation-exchange pre-column in Na^+ or Mg^{2+} form, followed by a separation on a ODS column coated with Zwittergent-3-14. In the first column all analyte cations were converted into common species in order to obtain a single peak per anion and then separated as ion pairs on the zwitterionic ion-exchanger.

Depending on the concentration of the sample, one or two peaks corresponding to one and the same ion pair can be observed [145]. The retention times for these peaks are almost equal and the peaks are not separated. At high concentrations the first peak with lower retention time dominates. The dilution of the sample leads to the equalisation of the areas of the two peaks. The second peak becomes dominant at low concentrations of the sample (less than 1 μ*M*). To explain this phenomenon, the authors used the Stern model of the electric double layer (EDL) [141] (Fig.1-4-11).

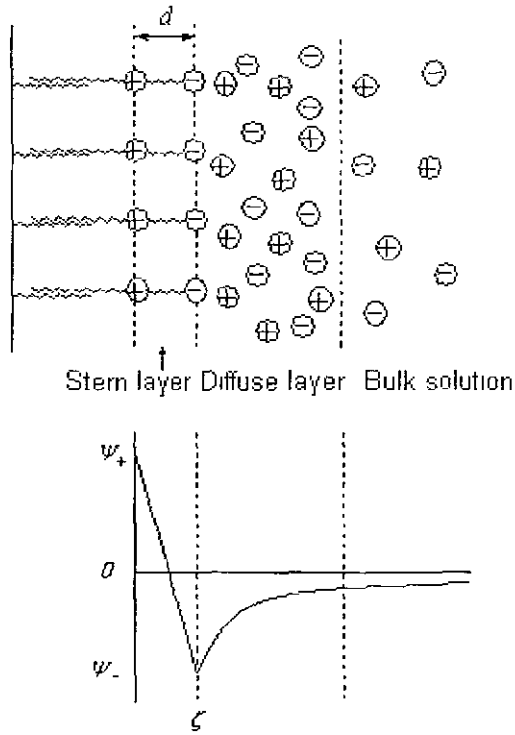


Figure 1-4-11. Schematic representation of adsorbed surfactant molecules and a profile of electrostatic potential. ψ - electrostatic potential, ζ - electrokinetic potential, d – distance between charges

The presence of oppositely charged groups on the surface of the zwitterionic stationary phase determines the low capacity of the EDL. When a sample with high concentration is analysed, it is not only the Stern layer that is saturated with ions, but also the diffuse layer, where the retention of ions is weaker. This leads to the occurrence of two peaks on the chromatogram, corresponding to one ion pair. The analyte ions eluted from the Stern layer were observed to have a slightly longer retention time than the analyte ions eluted from the diffuse layer. This difference in retention time becomes larger when both the analyte ions and the stationary phase exhibit hydrophobic properties. To obtain one peak on the chromatogram it is necessary to eject analyte ions into the diffuse layer, which is achieved by adding stronger retained ions to the sample. Unlike mono-functional ion-exchangers, the Stern layer of the zwitterionic stationary phase contains both cations and anions, so the analyte ions experience simultaneous forces of attraction and repulsion because of the close proximity of the positive and the negative functional groups on the stationary phase. The combined effects of the above result in an effective distribution of both the analyte cations and anions from the

electrical fields (stationary phase) to the bulk solution (eluent) without need for a displacing ion.

In summary, the use of water as an eluent in EIC enables a reduction in the limit of conductivity detection for ions, but at the same time does not allow the simultaneous separation of anions and cations as they elute as ion pairs. Furthermore, the absence of buffer properties in the case of pure water complicates the analysis of real samples. Switching to the electrolyte-containing eluent solves these problems and the relatively weak interactions between analyte ions and the zwitterionic stationary phase enables use of diluted eluents.

However, the model discussed above, as introduced by Hu *et al.* [141] was not universally accepted. The model did not consider that cation- and anion-exchange groups of the adsorbed surfactant could be located at different distances from the surface of the stationary phase. However, it is impossible to illustrate the precise location of surfactant molecules on the surface of ODS, as they take the form of different structures such as internal salts (especially without an electrolyte in eluent), intermolecular associates and micelles. Furthermore, this model did not explain the selective retention of cations or anions depending on the structure of the surfactant.

Using an electrolyte solution as the eluent was shown to increase selectivity and efficiency of the separation. Thus, the separation of $S_2O_3^{2-}$, NO_2^-, and NO_3^- on ODS coated with CHAPS became possible only with a phosphate buffer as the eluent [115]. Interestingly, when water was replaced with the electrolyte-containing eluent, the retention of anions on stationary phases coated with CHAPS and Zwittergent-3-14 increased [114,115,125]. The authors explain this phenomenon by stronger electrostatic interactions between analyte ions with the zwitterionic groups of adsorbed on the surface, when the electrolyte was used as the eluent, compared to those where the eluent was water. The retention of analyte ions increased until a certain concentration of the eluent was reached and any further increases did not further affect the ion-exchange capacity of the stationary phase. However, the retention of anions on ODS coated with ammonium sulphobetaine-1, where oppositely charged groups are separated by only one methylene group, decreased continually with the increase of the eluent concentration [124].

An alternative to traditional eluents based on inorganic salts are amino acids and zwitterions solutions [117,134,146]. Low background electroconductivity of these solutions at pH = pI provides high sensitivity conductivity detection. In work [134] – by

Macka and Haddad, histidine and 4-morpholineethanesulphonic acid (MES) solutions were used as eluents. MES is always zwitterionic in solution and cannot be present in cationic or anionic form. Histidine can be present in cationic, anionic or zwitterionic form and at pH = pI all forms are present in solution. Using such eluents it becomes possible to separate both anions and cations. However, selectivity to cations was relatively low. To increase the selectivity, an ODS coated with a mixture of zwitterionic (CHAPS) and anionogenic (sodium taurodeoxycholate) surfactants was used by Hu *et al.* [147]. Using such eluent simultaneous separation of 8 anions and cations (Fig 1-4-12).

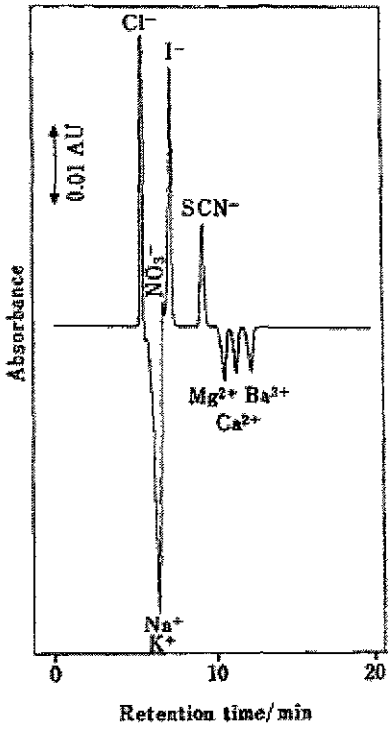


Figure 1-4-12. Simultaneous separation of inorganic cations and anions. Column: ODS-column 250 x 4 6 mm I.D., coated with NaTDC-CHAPS mixed micelles; mobile phase, 2.0 mM $CeCl_3$, flow rate, 0.7 ml/min; detection: UV at 253 nm. Reprinted from [147]

The retention mechanism for ions with an electrolyte solution as the eluent differs from that discussed earlier for EIC [135]. When inorganic ions are in contact with the immobilised surfactant, both cation–EDL and anion–EDL are formed. The cations are retained by the negatively charged groups of the zwitterionic surfactant to form a cation–EDL, the anions retained by positively charged groups of the surfactant form an anion–EDL. The configuration and properties of these type of EDL (binary–EDL [149]) (Fig. 1-4-13) are unusual compared to classical EDL.

In the binary–EDL, ions experience both attraction and repulsion forces simultaneously, which occurs due to cation- and anion-exchange sites located in close proximity on the single molecule of the zwitterionic surfactant. The authors of the

model [150] suggest that in the binary–EDL, cation and anions act as counterions for each other and are present in equimolar amounts. The results of simultaneous elution of the target cations and anion with water as an eluent provides evidence for this assumption. In the case of the binary-EDL, the EDL is formed by ions of the eluent, but not of the sample. The retention of anions is proposed to occur due to the formation of "temporary ion pairs" with cations from the EDL. It follows that the more stable the ion pair, the stronger the retention of the ion pair. Thus the retention of anions on the ODS phase coated with Zwittergent-3-14, using 1 m*M* $Ca(OH)_2$ as an eluent, is twice as strong as with 2 m*M* LiOH as an eluent [127]. The exception to this case is sulphate, which is weakly retained with both eluents. The increase of anion retention, corresponding to an increase of eluent concentration, is attributed by the authors to the gradual saturation of the binary-EDL. Sample cations cannot be retained on the stationary phase coated with the zwitterionic surfactant due to the repulsion from the positively charged outer surface of the binary-EDL.

Despite the fact that the above suggested mechanism does not contradict with experimental data, scope for further modifications of the model still exist.

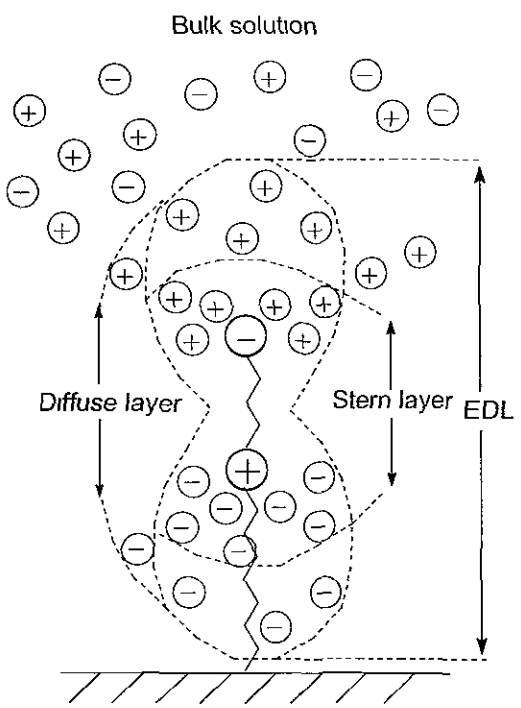


Figure 1-4-13. Schematic diagram of the adsorbed surfactant showing the binary electrical double layer established by eluent cations retained by the negatively charged functional groups of the zwitterionic surfactant and eluent anions retained by the positively charged functional groups of the zwitterionic surfactant

Okada and Patil [149] investigated mechanisms based on Poisson-Boltzmann theory. First was the partition model, based on the equality of chemical potential for thermodynamic phase equilibrium, where differences in the solvation of ions between

solution and the zwitterionic phases is assumed. Second was the ion-pair model based on the formation constants between ion-exchange groups on the surface and within ion pairs. The experimental results in this work showed unusual isotherms for small anions, where the analyte anions showed maximum retention at a particular concentration of the electrolyte-containing eluent, and where the retention of the analyte anions was affected by the nature of anions added to the eluent, but remained independent from the cation of the eluent. The developed models explained these experimental results by concluding that small and well-hydrated anions interact with the surface via a partition mechanism, while large and poorly hydrated anions interact via an ion pair mechanism.

All these models discussed above describe the retention of ions on an ideal stationary phase with homogenously separated zwitterionic molecules attached to the surface, without taking into consideration that surfactants are likely to form micelles. There have been a number of studies into the clarification of the alignment and structure of such surfactants adsorbed onto surfaces using atomic force microscopy [150-154], most of which focused on charged surfaces and cationic or anionic surfactants. In all reports it was shown that cylindrical or spherical hemi-micelle structures are formed on both charged and hydrophobic surfaces. For zwitterionic surfactants on hydrophobic surfaces, there is less information, even though the use of zwitterionic surfactants in micellar liquid chromatography is well established. However, on charged surfaces there are some very informative studies [153,155,156]. For example, the investigation of the adsorption of single chained surfactants *N*-(dodecyl-*N*,*N*-dimethylammonio)alcanoates [156] and double chained DDAB [153] on bare silica surfaces showed agglomerated micellar structures on the surface at concentrations above the known CMC.

It is known that zwitterionic micelles, as shown for 3-(*N*-dodecyl-*N*,*N*-dimethilammonio)propane-1-sulphonate (DDAPS), are electroneutral in pure water [157,158]. However, in electrolyte solutions a non-zero electrostatic potential emerges because of an imbalance between anion and cation partitioning It was therefore reported that the zwitterionic surfactant micelle obtains a negative surface charge and a positive inner charge [159-161]. The value of the ζ-potential on the micelle surface was found to be dependent on the concentration and nature of the anion of the electrolyte. At the same time the nature of the cation does not affect the micelle charge. For an explanation of this phenomenon two models were suggested. One was based on thermodynamic equilibrium distribution of ions between electrolyte and micelle phases,

and the second was based on the formation of ion pairs between charged micelle groups and electrolyte ions [162]. Interesting results were obtained in this work [149], where the distribution of ions between the electrolyte solution and the surface of ODS, dynamically coated with DDAPS, was studied. It was shown that the dependence of iodide retention on the concentration of NaCl in the eluent passes through a defined maximum. The same was observed for NaBr in the eluent, but not for $NaClO_4$, where an increase of eluent concentration lead to a decrease in anion retention. The influence of eluent concentration on the retention of iodide was found to decrease with the use of the following eluents:

$$NaCl < NaBr < NaClO_4.$$

The authors explain the increase in the retention of ions with increasing eluent concentration, on the change of the structure of the zwitterionic layer on the ODS surface. If zwitterionic molecules are flexible enough, *e.g* if they have a long intercharge chain, they are likely to form inter- and intra-molecular associates in the absence of an electrolyte in the solution, so the dipolar layer becomes thinner. With the increase of salt concentration in the solution, these interactions become weaker and the capacity of the dipolar layer increases, as it is shown in Fig 1-4-14 [161,163].

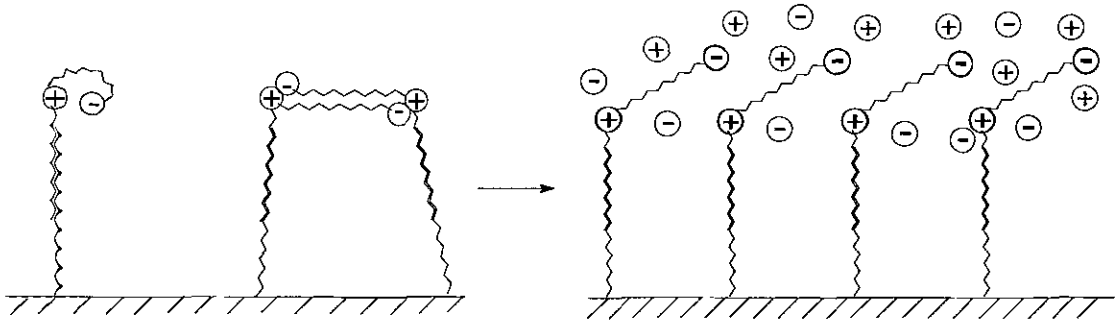


Figure 1-4-14. Schematic representation of adsorbed DDAPS molecules (A) in salt-free solution and (B) in salt solution

Chevalier *et al.* [161] found that because of stronger anion binding with the zwitterion than the cation, the adsorbed betaine surfactant molecules become charged, causing intermicellar repulsive effects and swelling of the zwitterionic layer as micelles, as shown in Fig 1-4-15.

From the analysis of the above studies, it is apparent that prediction of the exact structure of the adsorbed surfactant coating is non-trivial and as a result it becomes

complicated to accurately assess exact retention mechanisms. There are a number of parameters that would affect this structure when coating ODS surfaces with surfactants containing one or more weak ionic groups. For example, these include the degree of the residual silanols on the silica surface, the carbon loading of the silica and the pH and ionic strength of the eluent. The latter two parameters may, of course, vary during use of the coated phase and so continual rearrangement of the surface structure may also be possible.

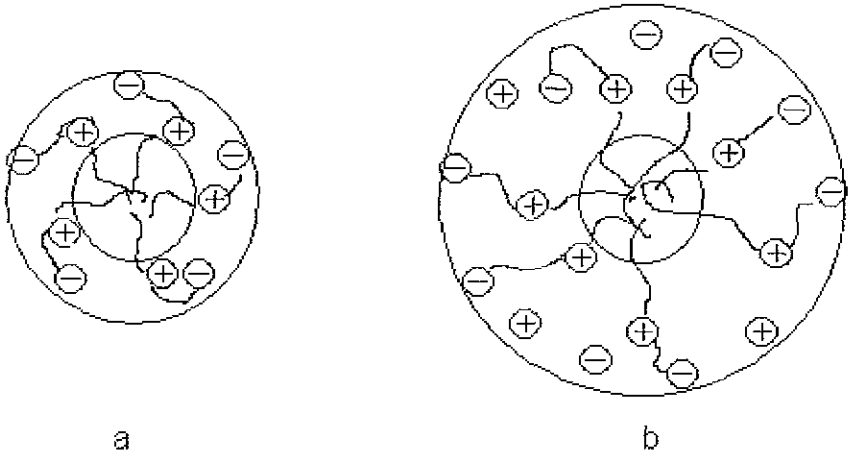


Figure 1-4-15. Schematic representation of the effect of salt adsorption on the micellar surface. The centre region represents the micellar core

The most recent retention mechanism was suggested by Cook *et al.* [164,165], comprising two simultaneous effects, one of ion exclusion and one of chaotropic interaction. The ion-exclusion effect arises as follows: the cation-exchange group on the outer part of the zwitterionic stationary phase contributes a negative charge that repels analyte anions by acting as Donnan membrane. However, the magnitude of this negative charge (and as a result, the degree of repulsion) varies depending on how strongly the mobile phase cations interact with the cation-exchange functional groups and how strongly eluent anions interact with quaternary ammonium groups. Strong interaction with the eluent cations decreases the surface negative charge, while strong interactions with eluent anions increase the surface negative charge. So the outer negative charge forms a barrier, which analyte anions have to pass through in order to interact with the anion-exchange sites of the zwitterionic stationary phase. Experimental data showed an increase in retention for all anions of interest with an increase in the

eluent cation charge, which shows that the nature of the eluent cations has an effect on the retention mechanism for anions.

The other part of the proposed mechanism concerns the interaction of sample anions with the quaternary ammonium functional group of the zwitterion. The experimental data showed that separation selectivity of ions followed the order of increasing chaotropic character. Chaotropic ions increase entropy of the system by disrupting the water structure surrounding these ions in solution. They are large with a low charge and have a low electron density. So the more chaotropic the analyte anion, the greater will be its retention. Also the authors confirm that the elution order of anions appears to be determined by "chaotropic selectivity" rather than by electrostatic effects.

* * *

Compared to traditional monofunctional stationary phases, zwitterionic ion-exchangers offer additional opportunities for varying separation selectivity. High efficiency, the ability to use highly diluted eluents, and also the possibility of simultaneous cation and anion separation, mark these stationary phases as very promising for further application in ion analysis. Nevertheless, the presence of oppositely charged groups located in close proximity within a single immobilised molecule, still provides challenges in the elucidation of exact retention mechanisms for inorganic and organic anionic and cationic species.

Table 1-5-2. Example separations on zwitterionic stationary phases

Stationary phase	Eluent	Analytes	Detection	Ref.
Polyampholyte ion-exchangers				
N,*N*-Dimethyl-*N*-methacryloyloxyethyl-*N*-(3-sulphopropyl) ammonium betaine (SPE)	water; 2.5 m*M* phosphate buffer, pH 7	KCl, KSCN, $Ca(SCN)_2$; Myoglobin, α-Chymotrypsinogen A, Cytochrome C, Lysozyme	Conductivity; UV 280 nm	[26, 28]
"Snake in the cage" – type stationary phases				
Retardion11A8; 23-46 μm	10 m*M* NH_4Cl	Zn^{2+}, Cd^{2+} preconcentration and separation		[35-37]
Retardion11A8; 23-46 μm	10 m*M* NH_4Cl	Zn^{2+}, Cd^{2+}	Gamma-ray spectroscopy	[38]
Amphoteric inorganic oxides (Al_2O_3)				
Spherical Alumina, 5 μm	100 m*M* NaCl,1mM acetate buffer	$I^- < Br^- < NO_3^- < NO_2^- < BrO_3^-$ < benzoate	UV 214 nm	[40]
Spherisorb Alumina Y	1 m*M* salicylic acid, 10 % CH_3CN, pH 5.8 0.5 m*M* sodium phthalate, 10 % CH_3CN, pH 5,2	$ClO_4^- < ClO_3^- < Cl^- < ClO_2^-$; $Na^+ < ClO_4^- < ClO_3^- < Cl^-$; $Na^+ < NH_4^+ < ClO_4^- < Cl^-$	UV 230 nm	[41]

Table 1-5-2. (continuation)

Multifunctional ion-exchange stationary phases				
IXSP 2, 3	CH_3CN-H_2O (2:1), 1 m*M* KH_2PO_4	Gly, Leu, Phe, Lys, Asp, Glu	UV 254 nm	[94]
Zwitterionic stationary phases with attached peptides				
ODS-BSA	0.5 m*M* phthalate buffer, pH 4.0	IO_3^-, Cl^-, NO_2^-, NO_3^-	UV 210 nm	[59]
BSA-80TS	0.15 m*M* tartaric acid, pH 3.6	IO_3^-, Br^-, NO_3^-, I^-, SCN^-	UV 210 nm	[60]
Centrally localised ion-exchangers with anion-exchange core				
KanK-Ast	2.4 m*M* Na_2CO_3-3.0 m*M* $NaHCO_3$	F^-, Cl^-, NO_2^-, HPO_4^{2-}, Br^-, NO_3^- SO_4^{2-}	Conductivity	[63]
KanK-ASR	3.0 m*M* Na_2CO_3-3.0 m*M* $NaHCO_3$;	F^-, Cl^-, NO_2^-, HPO_4^{2-}, Br^-, NO_3^- SO_4^{2-}	Conductivity	
KanK-BP	3 m*M* Na_2CO_3; 5 m*M* HNO_3	F^-, CH_3COO^-, $HCOO^-$, Cl^-; Na^+, NH_4^+, K^+	Conductivity	
Agglomerated ion-exchangers				
IonPac – AS4A	280 m*M* KCl, 1 m*M* EDTA, pH 2.5	Cr(III), Cr(IV)	PCR - luminescence	[65]

Table 1-5-2. (continuation)

IonPac – AS4, AS4A, AS5	HCl	Se(IV), Te(IV)		[72]
Dionex HPIC-CS5	0.5 m*M* $Ce(NO_3)_3$	Mg^{2+}, Sr^{2+}, Ba^{2+}, Cl^-, Br^-	UV 240 nm	[17]
IonPac CS5	6 m*M* pyridine-2,6-dicarboxylic acid	Ca^{2+}, Fe^{3+}, Cu^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+}, Mn^{2+}	UV 520 nm, PCR with PAR	[75]
Polyelectrolyte-coated stationary phases				
Silasorb C_8 - Ionene-2,5	2.6 m*M* potassium hydrogenphthalate, pH 7.0	IO_3^-, Cl^-, $H_2PO_4^-$, NO_3^-, SO_4^{2-} I^-, SCN^-, ClO_4^-	UV 260 nm	[78]
Silasorb C_8 – PHMG	1 m*M* potassium hydrogenphthalate, pH 6.0	F^-, Cl^-, Br^-, I^-, ClO_4^-, SO_4^{2-}	UV 260 nm	
Silasorb-S – Ionene-3-X	0.3 m*M* potassium hydrogenphthalate, pH 6.9	Cl^-, NO_3^-, I^-, SCN^-, ClO_4^-, SO_4^{2-}	UV 254 nm	[80]
Polysaccharide-coated stationary phases				
TSKgel IC-Anion-SW – Chrondroitin sulphate C	0.05 m*M* tartaric acid	IO_3^-, NO_2^-, Br^-, NO_3^-, I^-, SCN^-	UV 210 nm	[83]
TSKgel IC-Anion-SW - heparin	1 m*M* $CuSO_4$	Na^+, Mg^{2+}, IO_3^-, Ca^{2+}, Cl^-, NO_2^-, Br^-, NO_3^-, I^-, SCN^-	UV 200 nm	[85]
Poly(amino acid) functionalised stationary phases				
PolyCAT A	0.3 m*M* H_2SO_4–0.2 m*M* Li_2SO_4	Cl^-, NO_3^-, Na^+, K^+, Mg^{2+}, Ca^{2+}	Conductivity	[91]

Table I-5-2. (continuation)

Silica-based stationary phases with attached amino acids				
Silasorb 300 – glutamic acid	4 m*M* $HClO_4$	Mg^{2+}, Ca^{2+}, Mn^{2+}, Co^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}	Conductivity	[97]
KSK-L-hydroxyproline	5 m*M* sodium citrate	NO_2^-, IO_3^-, $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, I^-, ClO_4^-, SCN^-	Conductivity	[103]
Monolithic Performance-SI – lysine	1 m*M* phosphate buffer, 3 m*M* KCl	NO_2^-, BrO_3^-, Br^-, NO_3^-, I^-, SCN^-; Mn^{2+}, Co^{2+}, Cd^{2+}, Zn^{2+}	UV 214 nm UV 520 nm PCR with PAR	[109]
Silasorb 600 – histidine	5 m*M* oxalic acid	NO_2^-, $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, I^-, ClO_4^- SCN^-	UV 254 nm	[111]
Stationary phases with covalently bonded zwitterionic molecules				
Spheron 300 – DMAES	2.5 m*M* $NaClO_4$ 2 m*M* $HClO_4$	SO_4^{2-},Cl^-, F^-, NO_2^-, Br^-, NO_3^-; Li^+, Na^+, K^+, NH_4^+, Mn^{2+},	Conductivity	[96]
ODS dynamically coated with zwitterionic surfactants				
ODS-3-DDAPS	3 m*M* HCl	Br^-, Cl^-, NO_3^-	UV 210 nm	[135]
ODS-3-DDAPS	3 m*M* HCl	NO_3^-, NO_2^-, Br^-, Cl^-, SO_4^{2-}	UV 210 nm	[128]
ODS-Zwittergent 3-14	0.2 m*M* $NaClO_4$ - 0.3 m*M* Zwittergent 3-14	IO_3^-, NO_2^-, ClO_4^-, NO_3^-, BrO_3^-, I^-, SCN^-	UV 210 nm	[118]

Table 1-5-2. (continuation)

ODS-Zwittergent 3-14	Zwittergent 3-14	Cl^-, SO_4^{2-}, NO_2^-, Br^-, NO_3^-	UV 210 nm	[119]
ODS-Zwittergent 3-14	10 m*M* $Na_2B_4O_7$	SO_4^{2-}, Cl^-, NO_2^-, Br^-, NO_3^-, ClO_3^-, I^-	Suppressed conductivity	[166]
ODS-Zwittergent 3-14	40 m*M* H_2SO_4 to 1.0 m*M* $NaClO_4$ gradient	Br^-, NO_3^-, NO_2^-, I^-, SCN^-	UV 210 nm	[126]
ODS-Zwittergent 3-14	10 m*M* $NaHCO_3$ - 10 m*M* Zwittergent 3-14	SO_4^{2-}, Cl^-, NO_2^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-	Suppressed conductivity	[146]
ODS-Zwittergent 3-14	0.5 m*M* $Ca(OH)_2$ 2.0 m*M* LiOH	SO_4^{2-}, F^-,Cl^-, NO_2^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-	Suppressed conductivity	[127]
ODS-Zwittergent 3-14	7 m*M* H_3BO_3-$Na_2B_4O_7$ buffer	NO_2^-, Br^-, $NO_3^-ClO_3^-$	Conductivity	[167]
ODS-Zwittergent 3-14/TTA	20 m*M* Na_2CO_3	F^-, HPO_4^{2-}, Cl^-, SO_4^{2-}, NO_2^-, Br^-, NO_3^-	Suppressed conductivity	[131]
ODS-Zwittergent 3-14	H_2O	SO_4^{2-}, Cl^-, NO_2^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-	UV 210 nm	[148]
ODS-CHAPS	10 m*M* Na_3PO_4 – NaH_2PO_4, pH 6.8	$S_2O_3^{2-}$, IO_3^-, NO_2^-, NO_3^-, I^-, SCN^-; organic zwitterions	UV 230 nm or conductivity	[115]
ODS-CHAPS	10 m*M* $NaHCO_3$	SO_4^{2-}, Cl^-, NO_2^-, Br^-, NO_3^-,	UV 210 nm	[114]

Table 1-5-2. (end)

ODS-CHAPS ODS-CHAPSO	H_2O	Na^+, Ca^{2+}, Ce^{3+}	Conductivity	[116]
ODS-Ammonium sulphobetaine-1	10 m*M* NaCl	NO_2^-, Br^-, NO_3^-, I^-, SCN^-	UV 210 nm	[125]
ODS-Sodium taurodeoxycholate	5 m*M* $CuSO_4$	$S_2O_3^{2-}$, NO_3^-, I^-, SCN^-; Na^+, K^+	UV 210 nm	[122]
ODS- *N*-dodecylphosphocholine	30 m*M* KSCN	Ba^{2+}, Ca^{2+}, Mg^{2+}	Conductivity	[148]
ODS- Mitsubishi Reagent EF-700 ($C_8F_{17}SO_2NHC_3H_6N^+(CH_3)_2$-$C_2H_4$-$COO^-$)	1 m*M* H_2SO_4 4 m*M* HAc / 6m*M* NaAc	NO_2^-, $H_2PO_4^-$, Cl^-, Br^-, NO_3^-, ClO_3^-, I^-, SCN^-	UV 210 nm	[138]
Z1-Methyl (*N,N,N*-trimethylammonium butanesulphonate), Waters	10 m*M* Z1-Methyl	SO_4^{2-}, Cl^-, NO_2^-, Br^-, NO_3^-, ClO_4^-, I^-, SCN^-	Conductivity	[134]
Supelcosil LC-18-DB- DDMAA	150 m*M* KCl / 0.2 m*M* DDMAA	NO_2^-, Br^-, NO_3^-, I^-	UV 214 nm	[123]
Chromolith RP – DDMAA	10 m*M* KCl / 0.2 m*M* DDMAA	NO_2^-, Br^-, NO_3^-, I^-	UV 214 nm	[123]
Chromolith RP – DDMAA	10 m*M* phosphate buffer, 10 m*M* KCl, pH 3.0;flow gradient	NO_2^-, Br^-, NO_3^-, I^-, SCN^-	UV 214 nm	[124]
Onyx C_{18} capillary- DDMAU	0.5 m*M* phthalate, pH 4.0	BrO_3^-, NO_2^-, Br^-, NO_3^-, I^-, SO_4^{2-}, $S_2O_3^{2-}$, SCN^-, ClO_4^-	Contactless conductivity, C^4D	[139]

1.5. Conclusions

The information presented in the literature review on the properties and application possibilities of zwitterionic ion-exchangers show that these materials have a number of desired characteristics. Firstly, the combination of positively and negatively charged groups result in the reduction of shrinking and swelling of the stationary phase and an increase in mechanical stability of zwitterionic ion-exchangers. Secondly, mass transfer characteristics of surface-modified zwitterionic ion-exchangers can surpass those for ion-exchangers, where functional groups are distributed within the core. Two or more oppositely charged layers on the surface of the material prevent the penetration of ions inside the particle, which leads to fast mass transfer kinetics. Thirdly, the zwitterionic ion-exchangers exhibit the unique separation selectivity and frequent possibility of simultaneous separation of cations and anions on a single column. In addition, the presence of oppositely charged groups on the surface of the stationary phase can provide separation of zwitterionic analytes due to simultaneous interactions of the analytes with both functional groups of the stationary phase. Finally, the use of pure water as eluent is possible for some separations, which can improve the sensitivity of detection. Moreover, many of the zwitterionic ion-exchangers show similarities to biological species, such as amino acids and proteins and as a result, they can be used to obtain information about processes in biological systems.

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CHAPTER 2. EXPERIMENTAL

2.1. Instrumentation

In the current work the following HPLC systems were employed:

- Varian HPLC system comprising of a ProStar 230 pump, a ProStar 410 autosampler with a 100 μL sample loop and a ProStar 330 UV-detector (Varian Chromatographic Systems, Walnut Creek, CA, USA). For data acquisition, a Dell Optiplex GX-1 personal computer was used with Varian Star Chromatography Workstation V5.52 data acquisition software (Varian, Walnut Creek, CA, USA) installed.
- SpectraSYSTEM HPLC system comprising of a high pressure pump model P4000, vacuum degasser model SCM 1000, UV detector model UV2000 (AH Thermo Separations, San Jose, CA, USA) and a high pressure Rheodyne 7125 injector with a 20 μL sample loop (Rheodyne, Rohnert Park, CA, USA). For data acquisition, a Dell Optiplex GX-1 personal computer was used with Dionex AI-450 3.32 data acquisition software (Dionex, Sunnyvale, CA, USA) installed.
- Dionex DX-120 ion chromatograph (Dionex, Sunnyvale, CA, USA), which includes a pump, conductivity detector and injection valve with a 100 μL sample loop. For data acquisition, a RoverBook Voyager B415L laptop computer equipped with an ACD-16 data logger and PicoLog PWL044-1.8 data acquisition software (Pico Technology Ltd., Cambridgeshire, UK) installed.
- Dionex DX-500 ion chromatograph (Dionex, Sunnyvale, CA, USA), comprising of a GP50 gradient pump module, LC25 column oven, Rheodyne 7125 injector with a 25 μL sample loop (Rheodyne, Rohnert Park, CA, USA) and CD20 conductivity detector. For data acquisition, a Dell Optiplex GX-1 personal computer was used with PeakNet 6.60 SP1 data acquisition software (Dionex, Sunnyvale, CA, USA) installed.

For column thermostating a Mistral column oven (Spark-Holland, Emmen, Netherlands) was used. Samples were injected into chromatographic system using glass HPLC Microliter Hamilton syringe (Reno, USA) with 100 μL volume.

For measuring pH of solutions, Thermo Orion model 420 (Thermo Orion, Beverly, MA, USA) and RE 357 (EDT Instruments, Dover, UK) pH-meters were used

with a glass electrode, whilst the column outlet pH was monitored using a flow through pH electrode assembly.

2.2. Stationary phases

For the study of the retention of zwitterionic surfactants on RP sorbents the following columns and stationary phases were used:

- Chromolith RP-18e monolithic column, 100 x 4.6 mm I.D. (300 m^2/g surface area, 130 Å mesopore size, 2 µm macropore size; Merck KGaA, Darmstadt, Germany).
- Gemini C_{18} particle packed column, 50 x 2.0 mm I.D. (5 µm particle size, 375 m^2/g surface area, 14 % carbon load, 110 Å pore size; Phenomenex, Torrance, CA, USA).
- YMC-Pack ODS-2 particle packed column 150 x 4.6 mm I.D. (5 µm particle size, 17 % carbon load, 120 Å pore size; YMC Co. Ltd., Kyoto, Japan).

Separations were performed on:

- Gemini C_{18} particle packed columns, 4.0 x 3.0 mm, 50 x 2.0 mm, 50 x 4.6 mm, 100 x 4.6 mm I.D. (5 µm particle size, 375 m^2/g surface area, 14 % carbon load, 110 Å pore size; Phenomenex, Torrance, CA, USA), dynamically coated with each of the surfactants investigated.
- Chromolith RP-18e monolithic columns, 10 x 4.6 mm, 100 x 4.6 mm, 200 x 4.6 mm I.D. (300 m^2/g surface area, 130 Å mesopore size, 2 µm macropore size; Merck KGaA, Darmstadt, Germany) dynamically coated with each of the surfactants investigated.
- Onyx RP columns, 100 x 3.0 mm I.D. (300 m^2/g surface area, 130 Å mesopore size, 2 µm macropore size; Phenomenex, Torrance, CA, USA), dynamically coated with DDMAU.

DDMAU concentration was determined using:

- Supelcosil LC-18-DB particle packed column, 33 x 4.6 mm I.D. (3 µm particle size, 200 m^2/g surface area, 11 % carbon load, 120 Å pore size; Supelco, Bellefonte, PA, USA).

2.3. Reagents

All chemicals were reagent- or analytical grade purity.

Zwitterionic surfactants investigated for coating stationary phases were as follows:

- *N*-(dodecyl-*N,N*-dimethylammonio)undecanoate (DDMAU), *N*-(dodecyl-*N,N*-dimethylammonio)butyrate (both form Calbiochem, La Jolla, CA, USA), dodecylamine, didodecylamine, didodecyldimethylammonium bromide (DDAB) (Sigma-Aldrich, Gillingham, UK), dodecyliminodiacetic acid, dodecyldimethylamino acetic acid (synthesised in-house see below).

For preparation of eluents and solutions the following reagents were used:

- Salts: mo-, di- and tri- sodium phosphates (BDH-AnalaR, Poole, UK), sodium, magnesium, cerium chlorides and perchlorates (Fluka, Seelze, Germany), sodium citrate, sodium oxalate (Sigma-Aldrich, Gillingham, UK), ammonium acetate (Riedel-de-Haen, Seelze, Germany), calcium chloride, sodium sulphate, chlorate and perchlorate (Sigma-Aldrich, Gillingham, UK)
- Acids: glacial acetic acid, chloroacetic acid, citric acid, oxalic acid, phosphoric acid, hydrochloric acid (Sigma-Aldrich, Gillingham, UK).
- Bases: sodium hydroxide (Sigma-Aldrich, Gillingham, UK).
- Organic solvents: acetonitrile, methanol, ethanol (LabScan, Dublin, Ireland).

For the study of chromatographic properties of stationary phases of interest, the following reagents were used for standard preparation:

- Salts: sodium or potassium nitrate, nitrite, benzoate, iodide, iodate, thiocyanate, bromide, bromate, thiosulphate, 4-hydroxybenzoate, phthalate, acetate, dichloroacetate, trichloroacetate; lithium, sodium, potassium, ammonium, cesium, magnesium, calcium, strontium, barium chlorides, all obtained from Sigma-Aldrich (Gillingham, UK). Manganese, cobalt, lead, nickel chlorides and zinc and cadmium nitrates were obtained from BDH-AnalaR (Poole, UK).
- Nucleic acids precursors: adenine, guanine, citosysine, thymine, uracil (nucleobases); adenosine, guanosine, cytidine, uridine (nucleosides), adenosine-5'-mono-, di-, triphosphate, guanosine-5'-mono-, di-, triphosphate, cytidine-5'-mono-, di-, triphosphate and uridine-5'-mono-, di-, triphosphate (all Sigma-Aldrich, Gillingham, UK).

For the extraction of nucleotides, nucleosides and nucleobases from yeastolate samples perchloric acid, EDTA sodium salt and potassium hydrocarbonate were used, all obtained from Sigma-Aldrich (Gillingham, UK).

All reagent solutions were prepared using distilled deionised Milli-Q water from a Millipore water purification system (Millipore, Bedford, USA) with a specific resistance of 18.3 MΩ-cm. All mobile phases were filtered with a 0.47 μm Nylaflo

nylon membrane filter (LifeSciences, Steinheim, Germany) and degassed in an ultrasonic bath for 20 min prior to use.

2.4. Experimental techniques

2.4.1 Column modification

The required amount of the surfactant was weighed out for a stock solution of 5 m*M*, dissolved in Milli-Q water and then filtered. A 100 mL aliquot of this solution was then passed through the analytical column at a flow rate of 1.0 mL/min for 1 hour. Following coating, the column was washed with Milli-Q water prior to equilibrium with the desired eluent.

2.4.2. Synthesis of dodecyliminodiacetic acid

Synthesis was performed according to the method ofStain *et al* [1]. 5 mL of 10 *M* chloroacetic acid solution in ethanol was neutralised with 10 *M* NaOH ethanol solution to pH 9 followed by the addition of 5 mL of 5 *M* dodecylamine solution in ethanol. The resulting solution was allowed to stand for 3 days at room temperature, followed by 5 hours at 80-95 °C. The level of ethanol and pH were kept constant by addition of ethanol and NaOH. Acidification of the solution with concentrated HCl gave the crude product, which was purified by several recrystallisations from 95 % ethanol. White crystals were obtained and dried under vacuum.

2.4.3. Synthesis of (dodecyldimethylammonio)acetic acid

Synthesis was performed according to the method of Chevalier *et al.* [2]. A stiochiometric mixture of *N,N*-dimethyldodecylamine (technical grade, Fluka, Gillingham, UK) and sodium chloroacetate (Sigma-Aldrich, Gillingham, UK) was heated with reflux in methanol for 15 hours. The final compound was recrystallised from an acetone-methanol (90-10) mixture. White crystals were obtained and dried under vacuum.

2.4.4 Adsorption isotherms

The investigation of the adsorption of the surfactants on the reversed-phase materials was performed according to the method of Nikitin *et al.* [3]. Two series of DDMAU solutions with concentrations from 0.0 to 10.0 m*M* (n = 23) were prepared in pure water (DDMAU solution measured pH = 6.6) and in a 2 m*M* phosphoric acid

solution (pH 3.0). 10 mg samples of Gemini C_{18} sorbent were placed into test tubes and 2 mL of the appropriate DDMAU solution was added to each. The solution were shaken every 8 hours and left for sedimentation of the sorbent. After 72 hours the concentrations of DDMAU in the solutions above the sorbent samples were measured using RP-HPLC method with UV detection at 254 nm Column used for the determination of DDMAU was Supelcosil LC-18-DB 33 x 4 6 mm I.D., eluent used was 60 % MeCN – 40 % water, flow rate was 1.0 mL/min. The concentrations of supernatant solutions were determined using the calibration curves $S_{peak} = 2.894c + 1.335$, $R^2 = 0.998$ (for the determination DDMAU concentration at pH 6.6) and $S_{peak} = 3.061c + 4.186$, $R^2 = 0.995$ (for the determination DDMAU concentration at pH 3.0). Calibration standards were prepared in the range 0.0 – 10.0 m*M* and injected in triplicate.

2 4.5. Determination of the surfactant-coated column capacities

The determination of column capacity was a modified version of the experiment referred to by Hendricks *et al.* [4]. To evaluate the resultant amount of adsorbed DDMAU, an acid-base on-column titration procedure was applied, with the pH of the effluent monitored using a flow through pH meter. First, the column was coated with DDMAU and equilibrated with 5 m*M* sodium phosphate buffer, pH 3.0, to ensure that all carboxylic groups of the adsorbed surfactant molecules were protonated. The column outlet pH was recorded to ensure column equilibration at pH 3.0 was complete. A pH gradient was developed by washing the column with a 5 m*M* sodium phosphate buffer, pH 6.0 to 7.5. The pH gradient was performed first with the unmodified column in order to exclude the influence of dead volume and then again with the surfactant-coated column. The resulting capacity was estimated by subtraction of the first gradient from the second. To calculate the column capacity the system dwell volume should be known. The dwell time, t_{dwell}, is the time from the point of inception of the eluent step gradient to the tangent that extends through the inflection point of the curved area of the pH plot. The dwell volume, V_{dwell}, can be calculated by multiplying t_{dwell} by the eluent flow rate F.

$$V_{dwell} = t_{dwell} \times F \qquad \text{(eq. 2-4-1)}$$

2.4.6. Perchloric acid extraction of nucleotides

The extraction was performed according to the method suggested by Fish *et al.* [5]. A 0.7 ml volume of yeastolate sample was added to 0.7 mL of ice-cold 1.2 *M* perchloric acid and mixed The mixture was allowed to stand on ice for 5 min, centrifuged at 4000 g for 3 min. An 0.8 aliquot of the supernatant was added to 0.9 mL of ice-cold $KHCO_3$ / 50 m*M* Na_4EDTA in a 15-mL screw-top conical centrifuge tube and the contents were thoroughly mixed. After setting on ice for about 20 min the sample was centrifuged to pellet the $KClO_4$ and the aliquot of the supernatant was further diluted with water or rapidly injected.

2.5. Reference list

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CHAPTER 3. INVESTIGATION OF THE RETENTION MECHANISM ON REVERSED-PHASE SORBENTS COATED WITH *N*-(DODECYL-*N*,*N*-DIMETHYLAMMONIO)ALCANOATES

One of the easiest ways to obtain zwitterionic stationary phases is the dynamic modification of the surface of hydrophobic stationary phases with suitably hydrophobic zwitterionic or amphoteric molecules. Taking into consideration the complexity of the synthesis of stationary phases with covalently bonded zwitterionic molecules, and the ambiguity of chemical reactions taking place on the surface, the advantage of dynamically modified stationary phases is the simplicity of obtaining the stationary phases themselves and the homogeneity of ion-exchange sites. Additionally, the possibility of reapplication of the stationary phase coating allows the repeated regeneration of the column for extended use.

Amongst all the reported zwitterionic ion-exchangers, the most studied to-date are based upon ODS dynamically coated with zwitterionic or amphoteric surfactants. The most common modifiers have been sulphobetaine type surfactants containing a quaternary nitrogen atom and sulphonate group [1-3]. These surfactants have been predominantly used for the separation of anions and their selectivity to cations is limited. However, several mechanisms for the retention and separation of anions on sulphobetaine-type surfactant-modified stationary phases in ZIC have been proposed [1,2,4-6]. These mechanisms, such as the formation of "ion pairs" between oppositely charger ions in the solution [2], and the combined effect of ion-exclusion and chaotropic interactions [4], are described in Chapter 1.

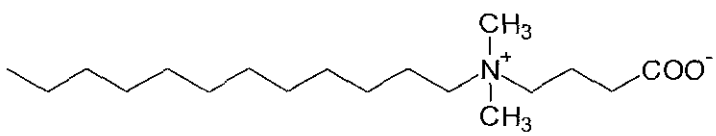
Recently, carboxybetaine type surfactants have been shown to exhibit equally unusual and variable selectivity [7-9]. However, no systematic studies of the retention mechanism have been carried out for RP substrates modified with carboxybetaine type surfactants.

The aim of this Chapter was the characterisation of zwitterionic/amphoteric coating on the surface of RP stationary phases and an investigation into the retention mechanism and selectivity for anions on a RP stationary phase, dynamically coated with *N*-(dodecyl-*N*,*N*-dimethylammonio)alcanoates with different intercharge chain length. This Chapter evaluates the difference in retention of anions, ion-exchange selectivity and separation efficiency between two such carboxybetaine type surfactants, and introduces a new cation charge gradient concept, previously unreported.

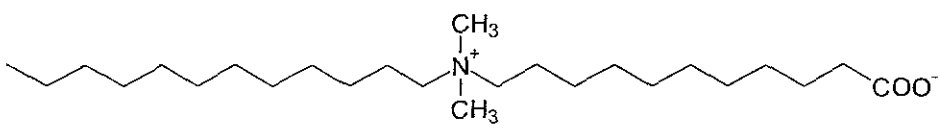
3.1. Selection of matrices and modifiers

3.1.1 Selection of modifiers

Ion-exchange properties of the zwitterionic stationary phase depend on the nature of ion-exchange groups and their positional relationship in the molecule. Another very important parameter is the hydrophobicity of the zwitterionic molecule, so that it may provide a stable surface layer. In the current work, zwitterionic surfactants *N*-(dodecyl-*N*,*N*-dimethylammonio)undecanoate (DDMAU) and *N*-(dodecyl-*N*,*N*-dimethylammonio)butyrate (DDMAB) were selected as modifiers. Both are amphiphilic carboxybetaine-type surfactants, which contain an inner quaternary ammonium group and a terminal carboxyl group, and differ with the number of methylenes in the intercharge link [10] (for structures see Fig.3-1-1). It has been shown [10] that increasing the number of intercharge methylene groups in carboxybetaine-type surfactants, increases the total hydrophobicity of the zwitterionic molecule, which tends to lower the critical micelle concentration (CMC), as well as causing the charge separation in the zwitterionic head group to grow, thereby increasing the strength of the repulsive interaction between head groups at the interface. Most of the references made to DDMAU and DDMAB in the literature have involved the utilisation of these surfactants in the extraction of viruses [11] and mycoplasma membrane protein antigens [12].



(a)



(b)

Figure 3-1-1. The structures of DDMAU and DDMAB

Prior to commencing this work, a number of possible zwitterionic surfactants were considered as stationary phase modifiers (Table 3-1-1). However, DDMAU was selected firstly based upon its considerable hydrophobic character, which would improve coating stability, and secondly due to the long intercharge link of the molecule, which would give a distinct terminal weak acid layer when adsorbed onto the stationary

phase surface. To evaluate the degree of surfactant adsorption on C_{18} RP matrices, the retention of DDMAU was determined on a range of C_{18} bonded stationary phases and compared to the retention of several other common ionic and zwitterionic surfactants, including those which were previously used in IC to coat RP columns, such as (dodecyldimethylamino)acetic acid [8,9] and didodecyldimethylammonium bromide (DDAB) [13,14]. Despite lower calculated hydrophobicity values, DDMAB was also chosen for study due to the similar structure, but different intercharge link, which may have an influence on the ion-exchange properties of the resultant zwitterionic stationary phase, once the surfactant is adsorbed onto the surface of the stationary phase. Table 3-1-1 shows the retention factors for three C_{18} RP columns including two columns used further in this study, namely, Chromolith Performance RP 18-e and Gemini C_{18}, for seven different surfactants using mobile phases containing 60 to 90 % of MeCN with 5 m*M* sodium phosphate buffer at pH 2.6. Direct UV detection was performed at 210 nm. The significant retention of DDMAU even at such high concentrations of MeCN illustrates the strong interaction of the surfactant with the C_{18} stationary phase, such that at MeCN concentrations below 60 % no peak was observed for DDMAU on the monolithic column or on the Gemini C_{18}. As expected, the retention of DDMAU was considerably higher than for (dodecyldimethylamino)acetic acid, dodecylamine, DDAB and DDMAB. The retention of surfactants observed, was in reasonable agreement with their calculated hydrophobicity values, or log P [15] (Table 3-1-1), which can therefore be utilised for general prediction of the stability of surfactant coatings on different RP materials.

Table 3-1-1. Retention factors (k) of ionic and zwitterionic surfactants on RP C_{18} columns

Surfactant	*k*					
	Column					
	Chromolith Performance RP-18-e, 100 x 4 6 mm I.D.		YMC Pack ODS-2, 150 x 2.4 mm I.D.		Gemini C_{18}, 5µ, 50 x 4.6 mm I.D	
	% MeCN		% MeCN		% MeCN	
	90 %	60 %	90 %	60 %	90 %	60 %
Dodecyliminodiacetic acid (log P = 2.34)	3.9	26 4	10.1	23.6	10.3	16.3
Dodecylamine (log P = 4.76)	7.9	48.6	19.0	43.6	15.5	65.7
(Dodecyldimethylamino)acetic acid (log P = 4.44)	2.5	15.8	4.9	16.6	8.3	55.7
Didodecylamine (log P = 10.63)	29.0	> 140	36.9	~ 106	76.7	99.0
DDAB (log P = 6.62)	0.9	4.5	3.3	6.1	2.3	4.0
DDMAU (log P = 6.89)	19.0	> 140	44.0	~ 128	-	~ 122
DDMAB (log P = 1.46)	1.1	9.1	2.8	8.3	2.5	5.7

3.1.2 Selection of stationary phase support

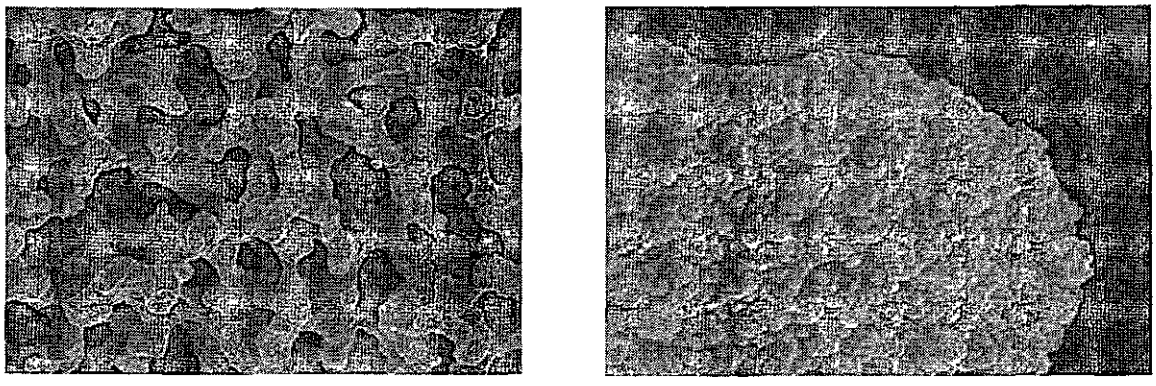
It is known that the stability of the surface coating strongly depends on the strength on interactions between stationary phase support and surfactant molecules. Aliphatic molecules are retained more strongly on ODS reversed-phases, rather than on polymer phases, which contain aromatic rings in their structure. So it was decided that two relatively new ODS sorbents, namely the silica-based monolithic columns Chromolith RP-18e and Onyx Monolithic C_{18}, and the silica-polymer hybrid phase Gemini C_{18}, would be investigated.

3.1.2.1. Monolithic reversed-phase columns

Particle packed columns have been used in chromatography for more than 30 years. The quality of such a column, i.e. its separation performance, is mainly determined by the particle size and distribution, and the quality of the packing of the particles within the column. In contrast to such packed columns, monolithic columns are made of a single piece of porous silica, which is also called a "silica rod". Monolithic columns can be prepared from both organic [16-23] and inorganic [24-29] polymers. The first monolithic columns were obtained in 1970s by Ross *et al.* [30] from polyurethane At the same time Knox and Bristow [31] suggested that the presence of large communicating pores would provide a network through which the mobile phase would flow and as a result, hydrodynamic flow could speed separations. At the same time, the surface area required for the adsorption and desorption processes could be obtained by creating mesopores on the monolithic skeleton's surface. The preparation of silica based monoliths progressed in the early 1990s with the invention of silica rod columns [24,29,32,33]. In 2000, the first commercially available product, the Chromolith column, was presented by Merck KGaA [34].

A synthetic route to silica monolith production was developed by Nakanishi and Soga [35] and applied by Tanaka [32]. It was based on hydrolysis and polycondensation of tetramethoxysilane in the presence of poly(sodium styrenesulphonate) or polyethylene oxide, which was used as template. A later method based on the packing of fused silica capillaries with ODS particles followed by thermal treatment to form monolithic columns was suggested by Horvath *et al.* [36]. After sintering, the monolithic column was octadecylated *in situ* with dimethyloctadecylchlorosilane. Further improvements of the technique used hybrid silanes [25]. The sol-gel process, described above [35], typically involves phase separation due to the polymer template

and sol-gel transition due to polycondensation of the silica precursor, which leads to a continuous silica network with a defined pore structure. Further thermal treatment results in monolithic silica columns with a bimodal pore structure consisting of macro- or through-pores and mesopores in the silica skeleton. A typical scanning electron microscopy picture of the porous monolithic silica is shown in Fig. 3-1-2. This method allows independent control of the pore size. It is possible to prepare monolithic silica columns with macropores in the range of 1 – 8 μm while keeping the size of mesoropores constant at 13 nm.



(a) *(b)*

Figure 3-1-2 SEM-pictures of (a) the typical porous structure with macropores (throughpores); (b) the mesoporous structure of the silica skeleton. Reprinted from [25]

This porous structure allows higher flow rates to be used in comparison to those possible for packed columns. It gives lower backpressure and thus the column itself, and the general system experiences less stress. At the same time it was reported that the lowest height of a monolithic theoretical plate was equal to 8 μm (approximately 125,000 *N*/m), which is similar typical to 3.5 μm particle packed columns [25,27,37], while Leinweber *et al.* showed that the performance of monolithic columns can be comparable to a column packed with 1 μm particles [38]. As long as the inlet bed settling is absent in these columns, the reliability, reproducibility and lifetime of the column is increased and the efficiency is maintained. Monolithic columns have been employed recently in IC for rapid separations of metal cations [39,40], inorganic anions [14,39,41], the simultaneous separation of anions and cations [42], and for the determination of hydrogen ions [43] and hydroxide [44].

3.1.2.2. Hybrid reversed-phase columns

Currently, many different RP, both silica- and polymer-based packed columns are commercially available. Silica-based RP columns exhibit high efficiency, but their

main disadvantage is relatively narrow pH stability (pH 2 – 8), which limits their possible application in ion-exchange chromatography. However, this instability is not so significant in the case of polymers, although usually they exhibit poorer efficiency, comparing to pure silica-based materials. Recently so-called "hybrid" or "twin" technology phases have been produced. This type of phase represents a combined silica and polymer matrix (hybrid) or silica core with a grafted polymer surface (twin). The latter phase (Phenomenex Gemini C_{18} column) exhibits mechanical stability and efficiencies of equivalent pure silica phases with additional pH stability over the range 1 - 12. [45]. These columns are currently used predominantly for pharmaceutical analysis [46,47].

3.2. Characterisation of the surface of Gemini C_{18} phase modified with DDMAU

Both volumetric and weight adsorption methods in static conditions are very common for the characterisation of sorbent surface properties [48,49]. By analysis of adsorption isotherms it is possible to make conclusions about the character and properties of the sorbent surface, and the nature of sorbent – sorbate interactions, as well as determining the main structural parameters of porous solid phases, such as diameter of pores, surface area and pore volume.

As long as dynamic modification or physical adsorption is used to obtain the zwitterionic ion-exchanger, it is very important to establish the structure of the coating (mono-layer, polylayer, or adsorbed micelles) formed on the surface of the sorbent. Previously, this was investigated for zwitterionic surfactants adsorbed on polar adsorbents [50-53] and to a lesser degree on hydrophobic substrates [54,55].

In order to understand the nature of the DDMAU coating on the surface of the RP substrate, adsorption isotherms of this surfactant on the Gemini C_{18} phase at pH 3.0 and 6.6 were obtained. Given the nature of the terminal weak carboxylic acid group (for DDMAU pK_a = 5.04 and DDMAB pK_a = 4.89, determined from potentiometric acid-base titration curves) it was expected that pH of the coating solution would result in different adsorption isotherms and resultant surface arrangements.

To find the adsorption isotherm of DDMAU, a known mass of adsorbent solid was shaken with a known volume of DDMAU solution at a given temperature until equilibrium. The concentration of surfactant in solution was determined and resultant adsorption isotherms were compared with known adsorption isotherms to elucidate a particular type of adsorption layer on the surface. The carboxybetaine adsorption

isotherms onto the Gemini C_{18} surface obtained by this procedure are shown in Figure 3-2-1.

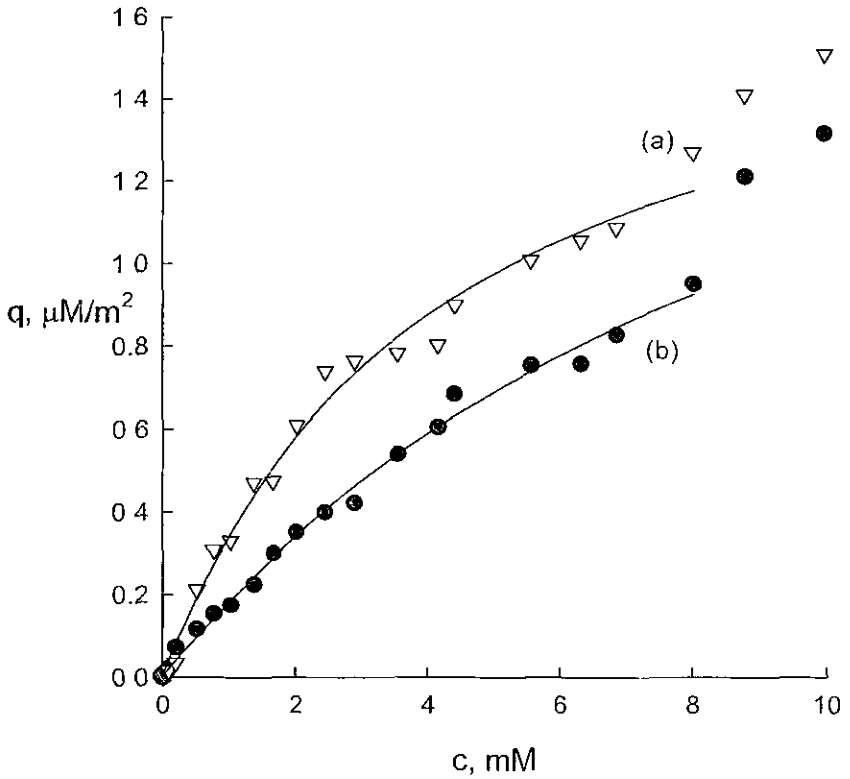


Figure 3-2-1. Adsorption isotherms of DDMAU on a Gemini C_{18} surface at (a) pH 6.6 and (b) pH 3.0 at 20° C. q – the rate of adsorption of DDMAU, c – the initial concentration of the surfactant

As it can be seen from the adsorption isotherm curves, at the concentration of the surfactant greater than 8 m*M*, a poly-layer adsorption starts. At surfactant concentrations less than 8 m*M* adsorption isotherms obtained at both pH 6.6 and 3.0 (Fig. 3-2-1) appear to obey the following Langmuir equation:

$$q = q_{\infty}\frac{Kc}{1+Kc} = \frac{\theta}{\theta_m}; \qquad \text{(eq. 3-2-1)}$$

where q – adsorption, K – equilibrium constant, c – concentration of the surfactant, q_{∞} - limit of adsorption, θ - number of occupied adsorption centers, θ_m – total number of adsorption centers [48]. As through the whole work concentrations of DDMAU less than 5 m*M* were used, it becomes possible to describe adsorption of the surfactant using Langmuir equation.

Both obtained adsorption isotherms curves can be described a by general formula, which is equivalent to the eq. 3-2-1:

$$f = \frac{bx}{1+ax} \qquad \text{(eq. 3-2-2)}$$

The parameters in eq. 3.2.2 were calculated (using Sigmaplot 2001 for Windows v.7.101 software) for the obtained adsorption isotherm experimental data. At pH 6.6 a –

0.240 ± 0.027, b = 0.430 ± 0.026, R^2 = 0.995, n = 14 and at pH 3.0 a = 0 094 ± 0.011, b = 0.202 ± 0.008, R^2 = 0.997, n = 14. Figure 3-2-1 shows the fitted curves to the two data sets obtained. The curve shape indicates that under the conditions investigated DDMAU molecules form a monolayer in up to 8 m*M* concentration in water solutions, as for example schematically represented in Fig. 3-2-2a. Some of the observed increase in adsorption at higher concentrations of the surfactant can be attributed to the beginning of multi-layer adsorption (Fig. 3-2-2b) or formation of different aggregates at the hydrophobic surface. Depending on the hydrophobicity of the surface and properties of zwitterionic surfactant, the formation of different aggregates is possible [55]. For example, the formation of spherical micelles on hydrophilic silicon nitride and mica has been reported, and hemi-cylindrical micelles of 3-(*N*-dodecyl-*N*,*N*-dimethylammonio)propane-1-sulphonate (DDAPS, CMC 3.0 m*M* in water) were observed on hydrophobic (graphite) surfaces. DDAPS also forms globular aggregates of defined periodicity of approximately 4.3 ± 0.3 nm on the flat hydrophobic surface of silicon wafers treated with trimethylchlorosilane, at concentrations 4.5 times higher that its known CMC value [54]. Therefore, it is also possible that under these conditions both DDMAU (CMC 0.13 m*M* [12]) and DDMAB (CMC 4.3 m*M* [12]) could form similar aggregates on the surface of the Gemini C_{18} RP material (Fig. 3-2-2c)

Using the Langmuir isotherm eq. 3-2-1 it is possible to find the limit of adsorption (q_∞) of DDMAU on the RP sorbents, adsorption equilibrium constant (K) and area per molecule at the solid phase-solution interface. This adsorption isotherm is plotted in linear coordinates c/q vs c using eq. 3-2-3.

$$\frac{c}{q} = \frac{1}{q_\infty} c + \frac{1}{q_\infty K} \qquad \text{(eq. 3-2-3)}$$

The slope gave the value of $1/q_\infty$ and its intercept gave the value of $1/q_\infty K$. With the known limit of adsorption, the area occupied by an adsorbed molecule is calculated using eq. 3-2-4.

$$S_0 = \frac{1}{q_\infty N_a} \qquad \text{(eq. 3-2-4)}$$

where N_a is Avogadro's number.

The adsorption equilibrium constant values for DDMAU adsorption at pH 3.0 and 6.6 were K = 0.383 ± 0.016 and K = 0.100 ± 0.004 respectively. The limit of adsorption of DDMAU on the Gemini C_{18} phase was found to be higher at pH 6.6 rather than at pH

3.0, which were q_∞ = 2.024 ± 0.162 $\mu M/m^2$ and q_∞ = 1.616 ± 0.065 $\mu M/m^2$, respectively.

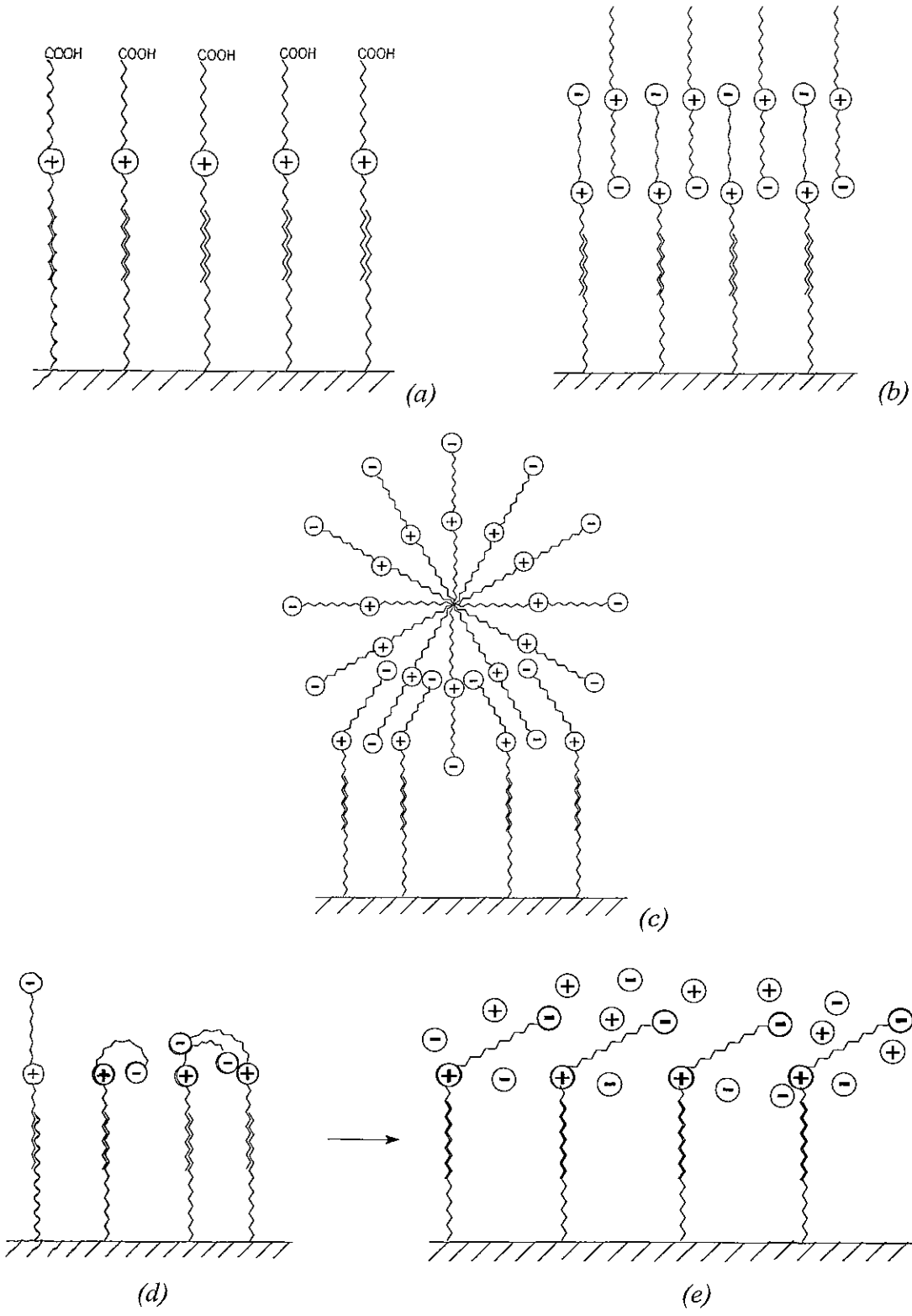


Figure 3-2-2. Possible structures of adsorbed DDMAU on a C_{18} reversed-phase surface, including (a) mono-layer at pH < 5, (b) double-layer at neutral pH, (c) possible adsorbed micelles, (d) internal salt interactions and (e) scheme of adsorbed DDMAU molecules in electrolyte-containing solution

The calculated area occupied by a DDMAU molecule at the surface was estimated as 102 ± 5 $Å^2$ at pH 3.0 and 82 ± 4 $Å^2$ for pH 6.0. This is in good agreement with the results obtained by Chevalier *et al.* [10]. The higher area per molecule in acidic conditions can also be attributed to the repulsion of uniformly charged molecules of surfactant adsorbed at the surface.

Obviously, under acidic conditions at pH < 4.0 the carboxylic groups of DDMAU molecules are protonated, so the formation of a monolayer on the surface of the sorbent is more probable, due to the repulsion between adsorbed charged molecules (Fig. 3-2-2a). At pH 6.6 DDMAU molecules can form internal salts or interact with oppositely charged groups of other adsorbed surfactant molecules (Fig 3-2-2b and 3-2-2d) with the potential of form multilayered structures, resulting in the higher observed adsorption limits. However, the formation of internal salts could also result in the decrease of apparent ion-exchange capacity at neutral pH. In either case, using eluents of relatively high ionic and/or of low pH, should result in the expected relatively rapid conversion of the surface to a uniform monolayer.

3.3. The investigation of retention mechanism

As mentioned in Chapter 1, several mechanisms for the retention and separation of anions in ZIC have been proposed for sulphobetaine-type surfactant – modified stationary phases [1,2,4-6,56,57]. These mechanisms involve concepts such as the simultaneous electrostatic interaction of analytes with oppositely charged sites within the zwitterion, the formation of "ion pairs" between oppositely charged ions in the solution [2], and the formation of a zwitterionic electrical double layer arising from the accumulation of oppositely charged eluent ions around the charges of zwitterion [57]. Additionally, specific retention mechanisms have been investigated using Poisson-Boltzmann theory, concluding that small well-hydrated ions interact with the zwitterionic surface by means of a partition mechanism, while large poorly hydrated ions interact via an ion-pair mechanism [5]. The most recent mechanism was based on two simultaneous effects, namely ion-exclusion and chaotropic interaction [6]. The ion-exclusion effect comes from the repulsion of analyte anions by the outer negative charges (adsorbed sulphobetaine molecules). This repulsion effect could be either increased or decreased depending on the nature of the analyte ion and the shielding of charges of the zwitterionic molecule by anions and cations added to the eluent as a buffer or ionic strength regulator. Retention is therefore determined by the ability of an analyte to directly (chaotropically) interact with the inner positive quaternary

ammonium group, and to compete with the corresponding eluent anion for that interaction.

However, despite the availability of the above studies, no such systematic investigation has been carried out for RP substrates modified with carboxybetaine type surfactants. Therefore, the main focus of the current study is an investigation into the retention mechanism and selectivity for anions on the RP sorbent, dynamically coated with *N*-(dodecyl-*N*,*N*-dimethylammonio)alcanoates of varying chain lengths. This section evaluates the difference in retention of inorganic and organic anions, ion-exchange selectivity and separation efficiency between DDMAU and DDMAB-coated stationary phases.

3 3.1. Effect of coating solution ionic strength on the capacity of stationary phases

Increasing the ionic strength of the coating solution may cause either an enhancement in adsorption of zwitterionic surfactants due to a "salting out" effect, based upon reduced electrostatic repulsion and closer packing of the surfactant molecules upon the surface, or a possible reduction in relative adsorption due to a decrease in the monomolecular concentration of the surfactant in the solution resulting from lower CMC values. For example, Patil and Okada noted the drop in CMC values for DDAPS from 3.0 m*M* in pure water to 2.0 m*M* in 0.05 *M* sodium perchlorate or in 0.4 *M* sodium chloride [58]. However for the combined reasons mentioned above, the observed cumulative effect of the addition of sodium chloride to the coating solution at 0.1 *M* concentration caused less than 5 % reduction in the amount of DDAPS adsorbed on ODS surface.

To study the effect of ionic strength on the adsorption of DDMAU, in this case the particle packed RP (Gemini C_{18}) 4 x 3 mm I.D. column was coated using a series of coating solutions according to the procedure described in Section 2.4.6. The concentration of the coating solutions was 5 m*M* DDMAU and the concentration of sodium chloride used to increase ionic strength varied from 0 to 100 m*M*. From the pH gradient curves using eq. 2-4-1, the V_{dwell} value was calculated and found to be 1.2 mL (at 1.0 mL/min flow rate). Repeating this experiment with DDMAU columns modified at different ionic strength allowed calculation of the buffering capacity for columns coated under different conditions. A comparison of the column eluate pH of an unmodified column with five columns coated at different ionic strengths can be seen in Fig. 3-3-1. The obtained results (Fig. 3-3-2) showed significant increases in capacity with increasing ionic strength of the DDMAU coating solution, up to 10 m*M* NaCl

followed by a more gradual increase between 10 and 100 m*M* resulting in an effective capacity range from 45 - 120 μmol.

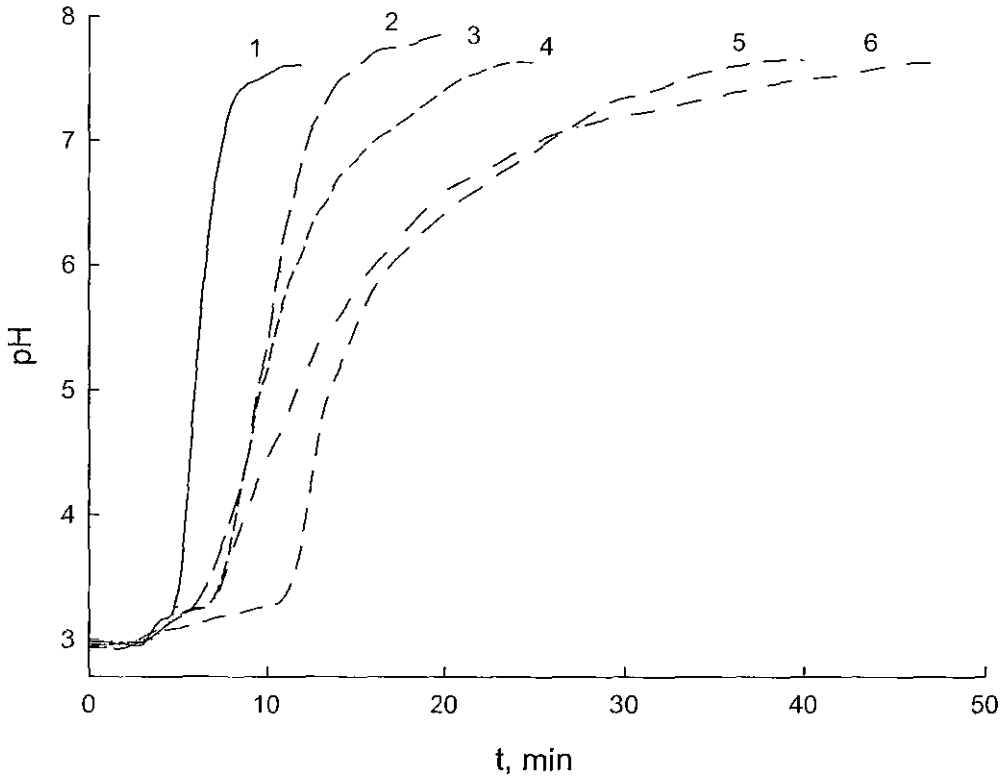


Figure 3-3-1 A comparison of the column eluate pH using a linear pH gradient for an unmodified column with coated columns. pH gradient profiles for Gemini C_{18}:
1 - uncoated column, 2 - 5 mM DDMAU in water, 3 - 5 mM DDMAU in 1 mM NaCl, 4 - 5 mM DDMAU in 10 mM NaCl, 5 - 5 mM DDMAU in 50 mM NaCl, 6 - 5 mM DDMAU in 100 mM NaCl modified columns.
Eluents: A – 5 mM phosphate buffer, pH 3.0; B – 5 mM phosphate buffer, pH 7.6.
Linear pH gradient: 0 0 – 1.0 min 100 % A to 100 % B

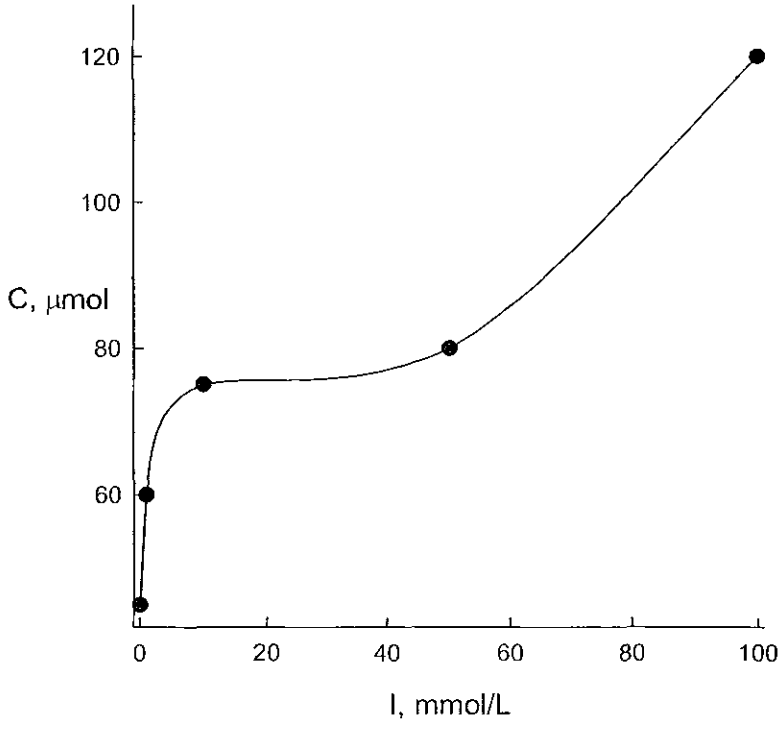
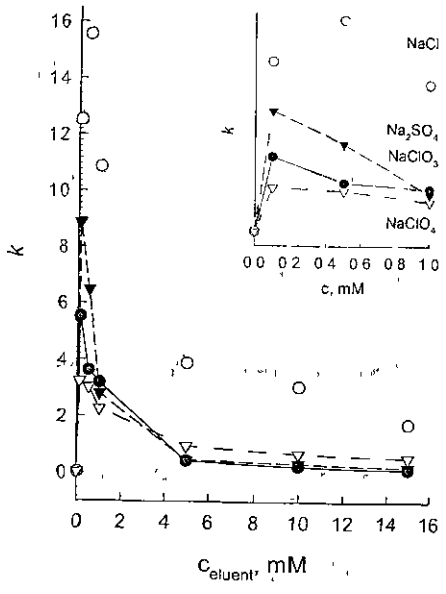


Figure 3-3-2. Determined column exchange capacities (H^+) resulting from increasing ionic strength of column coating solution

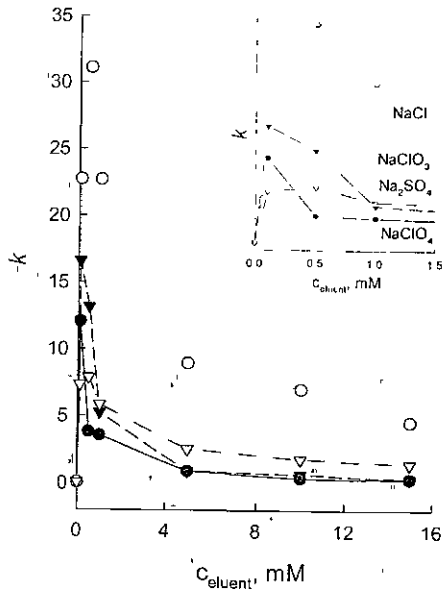
3.3.2 The effect of eluent concentration on retention of anions

The anion exchange properties of particle packed and monolithic RP columns coated with DDMAU and DDMAB (all coated at pH 6.6, no salts added) were studied. The sodium salts of chloride, chlorate, perchlorate and sulphate were chosen as the eluents due to their UV transparency. The retention data for nitrite, nitrate, bromide, iodate and iodide were collected for a series of eluents of concentration ranging from 0 to 15 m*M*. The effect of altering the concentration of each of the above eluents upon the retention, for particle packed and monolithic RP columns coated with DDMAU are shown in Fig.3-3-3 and Fig 3-3-4, respectively. Fig.3-3-5 and Fig.3-3-6 show this effect for particle packed and monolithic RP columns coated with DDMAB. Interestingly, a very clear point of maximum retention for eluent concentrations of approximately 0.1 m*M* was recorded for all eluent salts and was observed for both coatings on all columns.

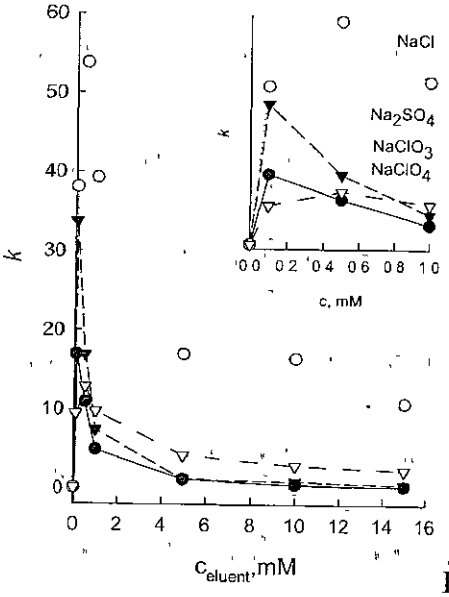
In previous studies investigating DDAPS coated RP columns, analyte retention times were seen to either initially increase (as compared to the retention obtained in pure water) when a weakly retained anion (*e.g* , SO_4^{2-}, Cl^-) was added to the eluent, or decrease when a strongly retained anion like ClO_4^- was used, reaching the point where further increases of eluent strength showed little effect upon retention [56]. In the current study, the sharp increase in retention of anions with the change from pure water to the low strength eluents, and the subsequent decrease in retention with further increases in eluent



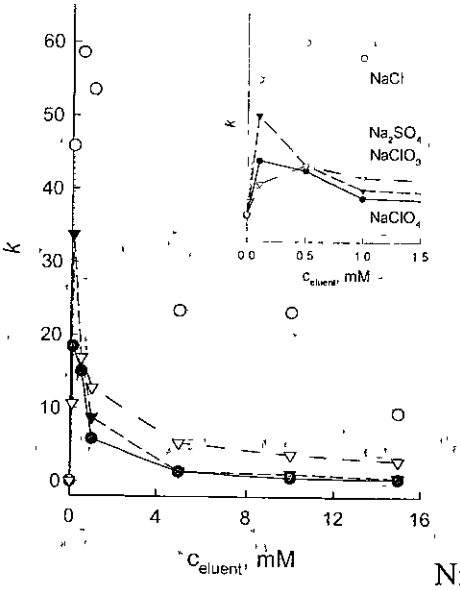
Iodate



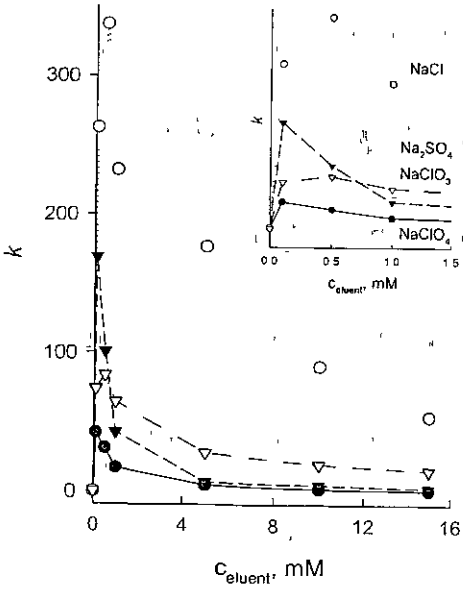
Nitrite



Bromide

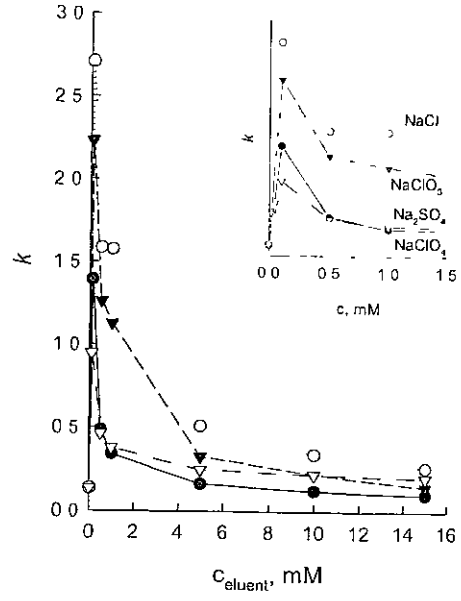


Nitrate



Iodide

Figure 3-3-3. Effect of concentration of sodium salts within the eluent on the retention of different anions on a Gemini C_{18} column coated with DDMAU, 50 x 4.6 mm I.D.



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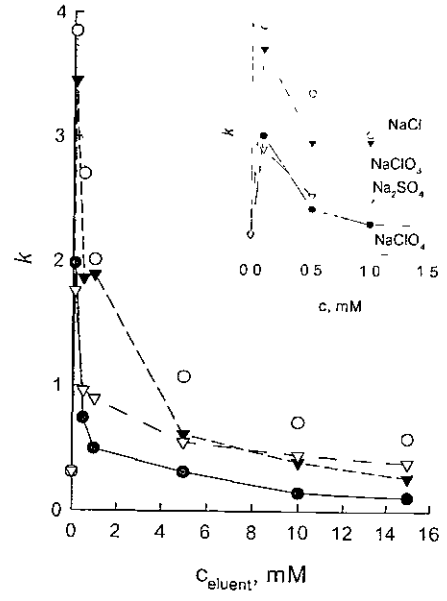
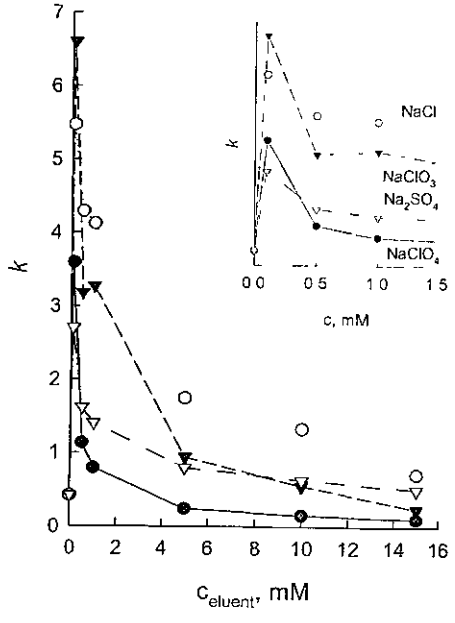


Nitrite



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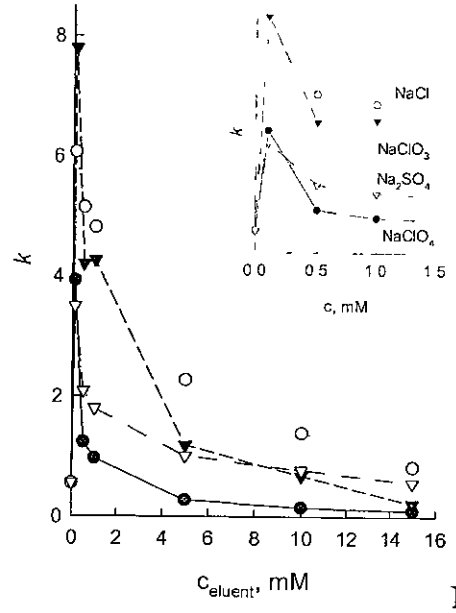
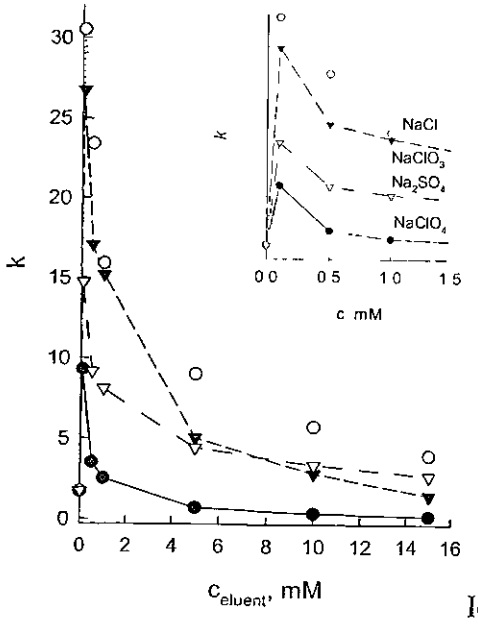
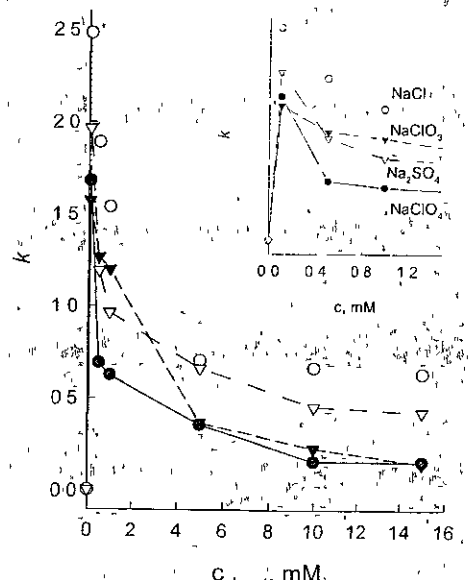


Nitrate

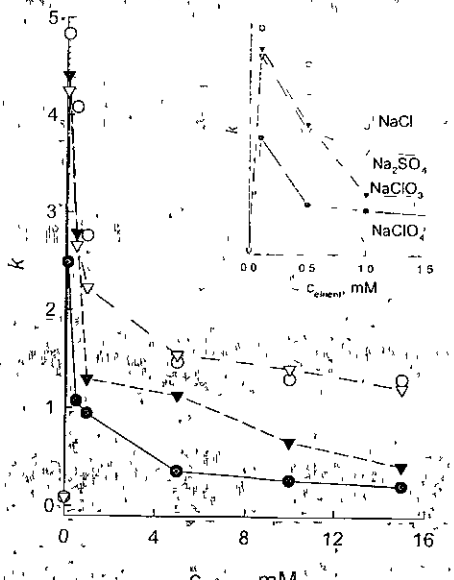


Iodide

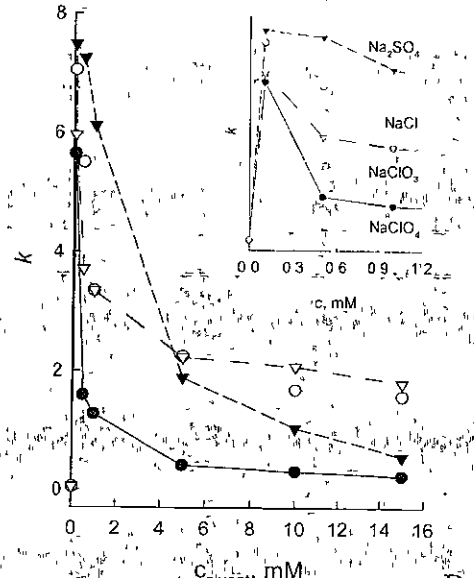
Figure 3-3-4. Effect of concentration of sodium salts within the eluent on the retention of different anions on a Chromolith RP column coated with DDMAU, 100 x 4.6 mm I D



Iodate



Nitrite



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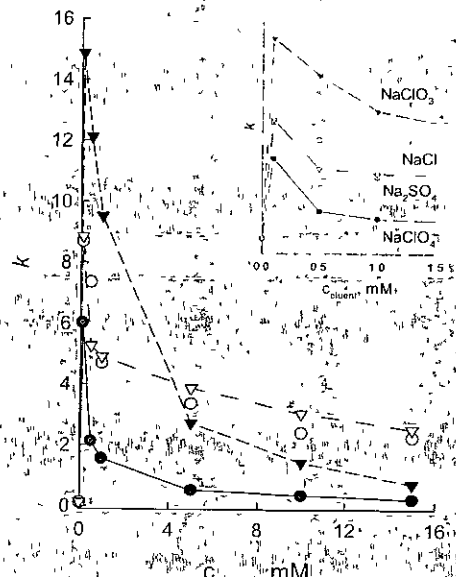
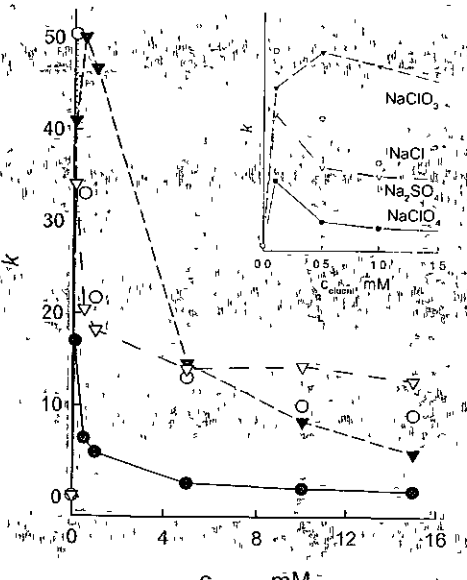
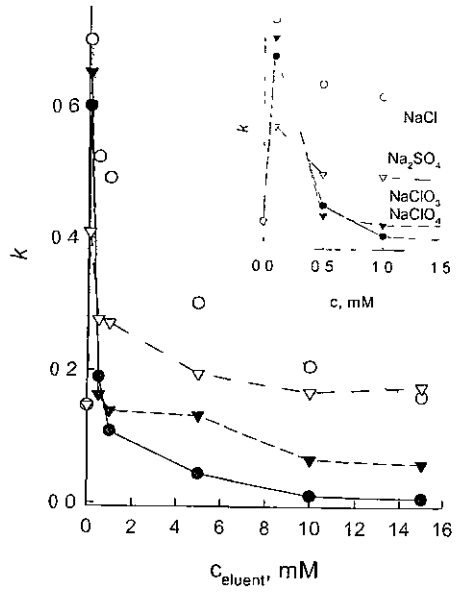


Nitrate

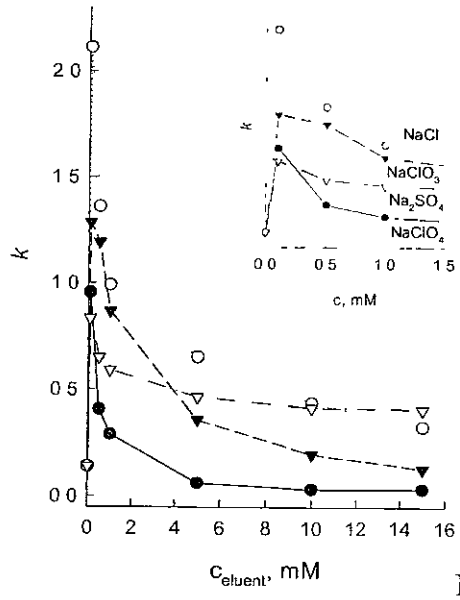


Iodide

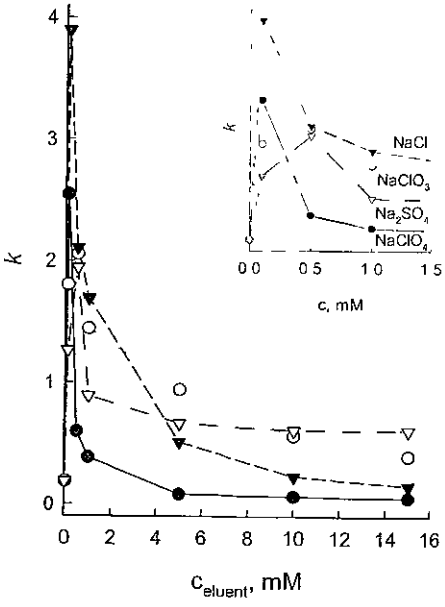
Figure 3-3-5: Effect of concentration of sodium salts within the eluent on the retention of different anions on a Gemini C_{18} column coated with DDMAB, 50 x 4.6 mm I.D.



Iodate



Nitrite



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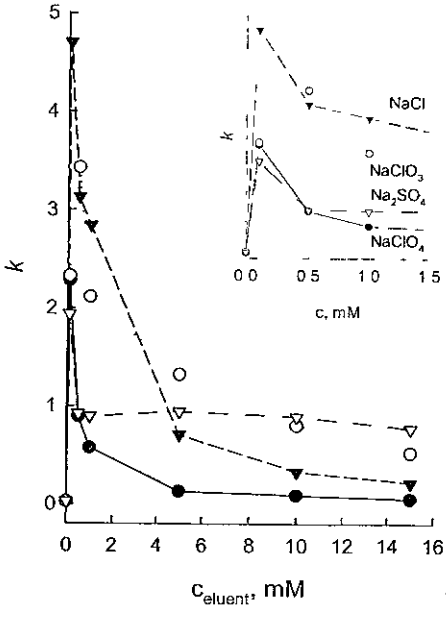
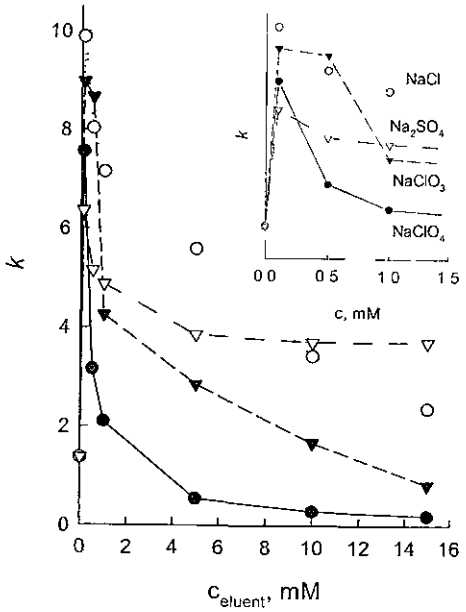


Nitrate



Iodide

Figure 3-3-6. Effect of concentration of sodium salts within the eluent on the retention of different anions on a Chromolith RP column coated with DDMAB, 100 x 4.6 mm I D.

concentration can be explained as follows. It is predicted that the carboxybetaine molecules adsorbed at the surface remain relatively mobile and flexible. This is assumed, since was shown earlier, that for a completely coated surface with a homogeneous monolayer distribution of the adsorbed molecules, that the spacing between them was approximately 10.2 Å (for DDMAU). This spacing is approximately the same as the length of the intercharge arm between $-N^{+}(CH_3)_2-$ group and $-COO^{-}$ group which is 9.9 Å [5]. This makes the molecules on the surface flexible enough for the formation of both internal salts and for the other possible changes in molecular configuration as shown in Fig. 3-2-2d.

In the case of DDMAB, the length of the intercharge arm is only 5.2 Å, while the distance between molecules is 11.4 Å [10]. Both intermolecular and intramolecular (provided the molecule intercharge arm is flexible enough to form stable internal salts) interactions could take place between positive and negative groups within the surfactant molecules, but these become significantly weaker with increasing salt concentration in the eluent. This means that the dipolar layer is missing or substantially reduced in pure water and appears immediately with the increase of eluent ions. Similar changes in dipolar layer have been Reported by Chevalier *et al.* [59-61], who found that because of stronger anion binding with the zwitterion than for cations, the adsorbed surfactant betaine molecules become charged, causing intermicellar repulsive effects and swelling of the zwitterionic layer. They also showed that this effect increases with an increase of intercharge arm length and explained it by electrostatically driven "reorganisation". Based on this, it can be assumed that the nature of the eluent anion is significant, as the stronger the anion binds to the zwitterionic molecule, the stronger intermicellar repulsion is, and as a result, interactions between positive and negative parts of the surfactant molecule become weaker, making formation of internal salts less probable. Following this initial surface reorganisation effect, with a future increase of eluent concentration the expected ion exchange dominates and retention continually decreases.

3.3.3. Effect of eluent cation charge on analyte retention

According to the results of Cook *et al* [56], who studied the ion-exchange mechanism for ODS substrates coated with sulphobetaine surfactants, the retention mechanism consists of two parts: ion exchange and chaotropic interactions. In the current study, the carboxylic acid groups on the external part of the zwitterionic layer possess a negative charge, which repels analyte anions by acting as a Donnan membrane. The magnitude of this negative charge depends on the strength of the

interaction between cations from the eluent and this weak acid group, as well as on the strength of interaction between anions and the internal quaternary ammonium groups. Strong interaction with eluent cations decreases the surface negative charge, while strong interaction with eluent anions increases the surfactant negative charge. So the outer negative charge forms an effective barrier through which the analyte anions must penetrate in order to reach the quaternary ammonium functional group (the interaction of analyte anions with the quaternary ammonium functional group of the carboxybetaines depends also on the chaotropic character of the anion). Ignoring the obvious significance of eluent pH, which will be discussed later in Section 4.2, this charge barrier can be lowered by partial neutralisation using 2+ and 3+ cations within the eluent, resulting in an observed increase in retention for analyte anions.

To study the above cation charge effect upon the retention of anions on the carboxybetaine coated columns (particle packed and monolithic RP columns coated with DDMAU and DDMAB), the use of sodium, magnesium and cerium chlorides and perchlorates as sources of mono-, di- and triply charged cations within the eluent were investigated. 10 m*M* eluents with an approximate pH 6.0 – 6.6 were prepared ensuring that the carboxylic acid groups of the carboxybetaines were fully dissociated. Again column outlet pH was monitored to ensure column equilibration was complete. In the case of eluents prepared from both chloride and perchlorate salts, the increase in the cation charge within the eluent caused an increase in the retention for all of the analyte anions investigated, except iodide, for which maximum retention was observed with Mg^{2+} salts. This effect was observed for both, perchlorate and chloride based eluents and all carboxybetaine coated columns: particle packed RP coated with either DDMAU or DDMAB (Fig. 3-3-7), and monolithic RP coated with the same surfactants (Fig. 3-3-8). The reason for the atypical behavior of iodide may be the formation of an iodide complex with cerium [62], hence causing a reduction in retention. For all columns, particle packed and monolithic RP coated with both DDMAU and DDMAB, similar dependences were observed, the only difference was lower *k* values in case of DDMAB coated columns. This was due to the lower hydrophobicity of DDMAB compared to DDMAU, which resulted in a smaller amount of adsorbed DDMAB and a lower ion-exchange capacity of the corresponding columns. The corresponding values of log P are shown in Table 3-1-1.

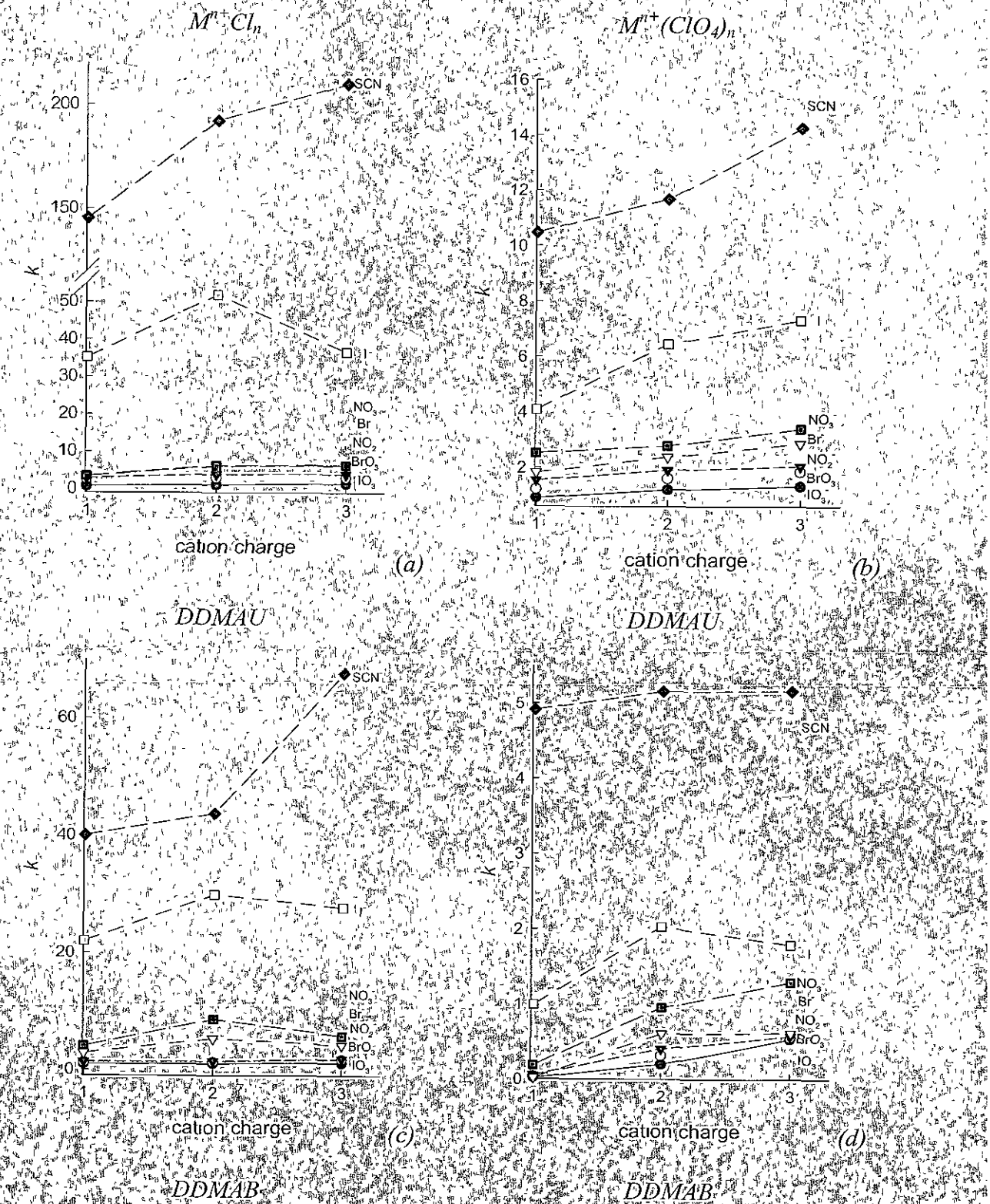


Figure 3-3-7. Effect of the eluent cation charge on the retention of analytes using eluents comprising 10 mM (with the respect to the cation) chloride (a, c) and perchlorate (b, d). Cation: 1 – Na^+, 2 – Mg^{2+}, 3 – Ce^{3+}. Column: Gemini C_{18} coated with DDMAU (a, b), and DDMAB (c, d), 50 x 4.6 mm I.D.

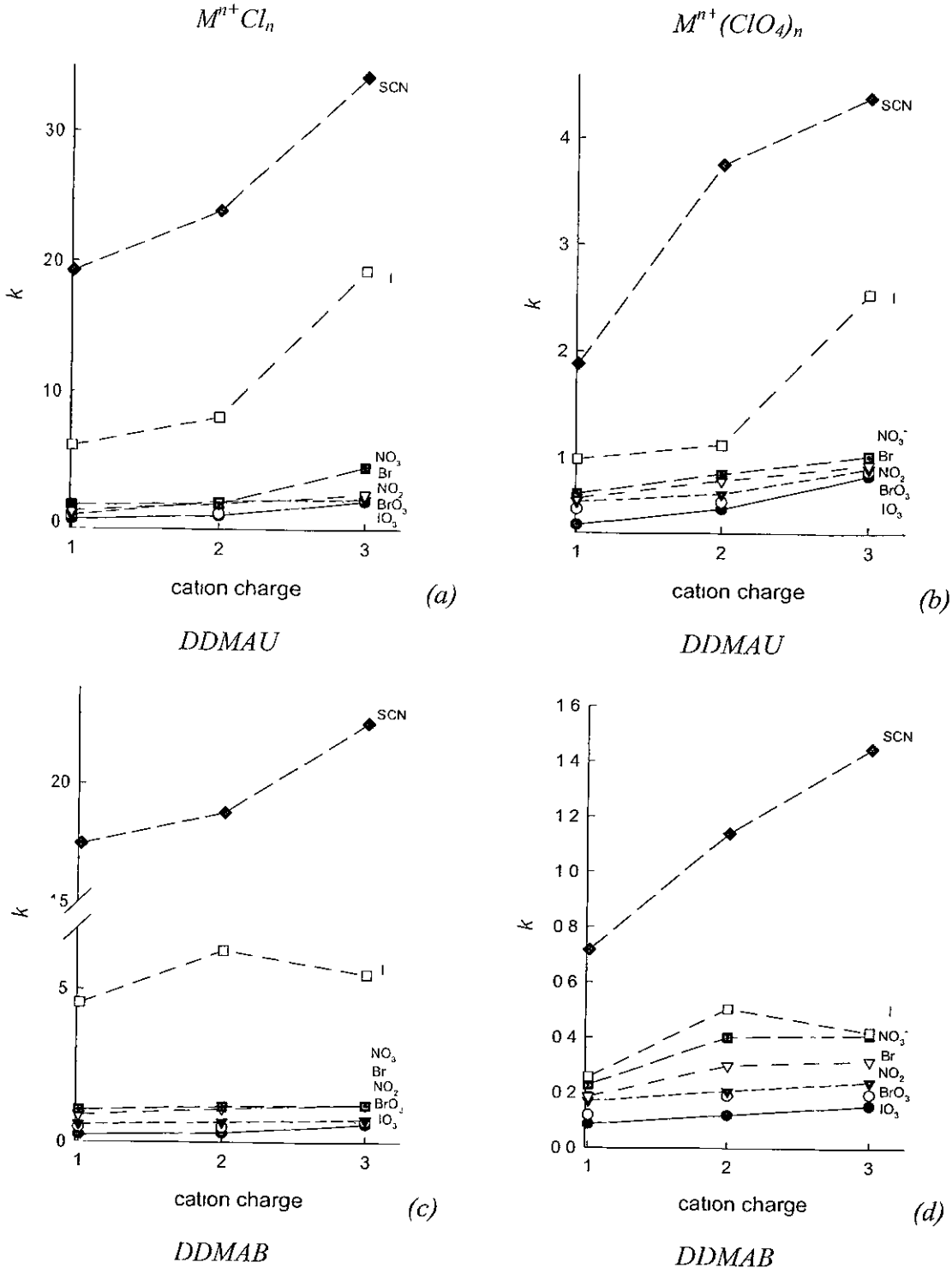


Figure 3-3-8 Effect of the eluent cation charge on the retention of analytes using eluents comprising 10 mM (with the respect to the cation) chloride (a, c) and perchlorate (b, d). Cation. 1 – Na^+, 2 – Mg^{2+}, 3 – Ce^{3+}. Column: Chromolith RP coated with DDMAU (a, b), and DDMAB (c, d), 100 x 4 6 mm I D.

There was a weak interaction between Na^+ and the carboxylic acid group of adsorbed surfactant and a strong interaction between ClO_4^- and the internal quaternary ammonium group when investigating the 10 m*M* $NaClO_4$ eluent. This led to the

formation of a strong negatively charged Donnan membrane (Fig. 3-3-9b), which exerts a strong repulsive effect on analyte anions. When the 10 m*M* $CeCl_3$ eluent was used, a strong interaction between Ce^{3+} and the carboxylic acid group and a weak interaction between Cl^- and the quaternary ammonium group took place (Fig. 3-3-9c). In this case, the Donnan membrane becomes weakly positively charged, which results in stronger retention of analyte anions.

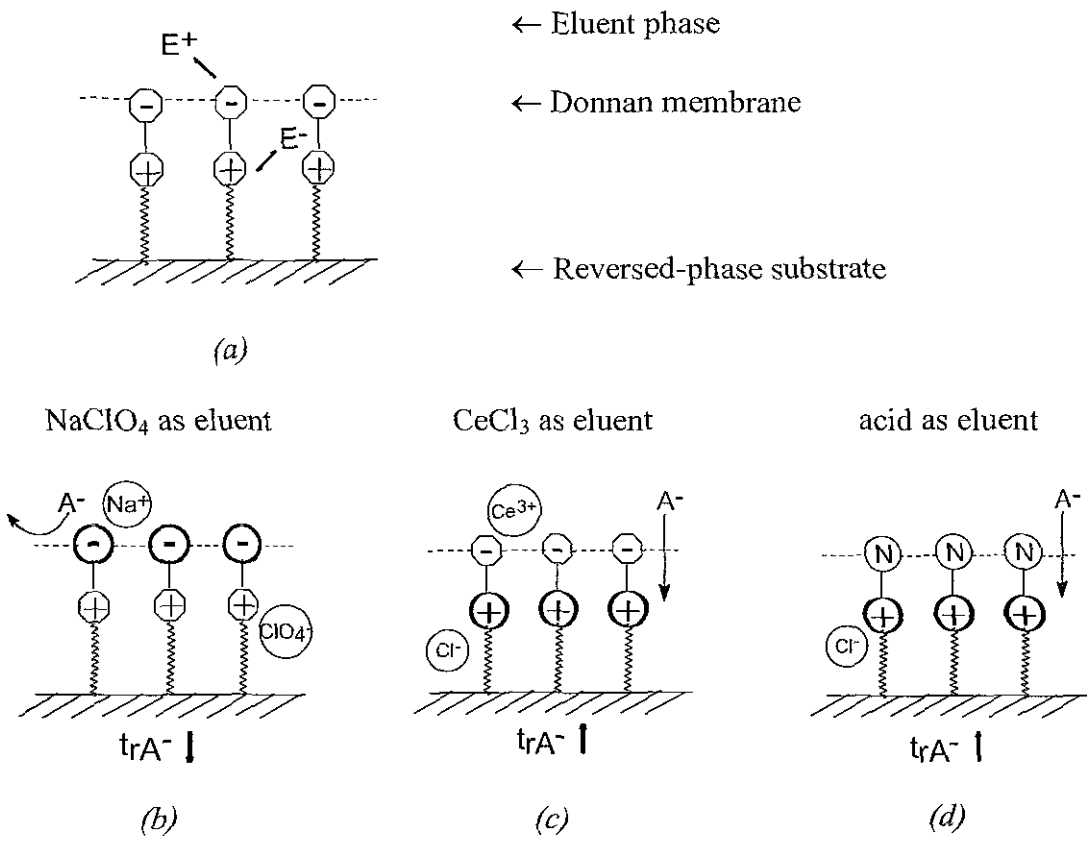


Figure 3-3-9. Proposed schematic representation of retention mechanism. (a) Establishment of Donnan membrane, (b) use of $NaClO_4$ mobile phase, (c) use of $CeCl_3$ mobile phase, (d) use of low pH eluent. This schematic diagram is adapted from [56]

This mechanism also explains the strong anion retention seen in acidic eluents. It has been shown previously for similar carboxybetaine type surfactants that the pH of the eluent strongly affected the retention of anions [8] (see Section 4.2). As the DDMAU and DDMAB molecules contain a weak acid terminal group, a decrease of eluent pH reduces the number of dissociated carboxylic acid groups and hence significantly increases anion exchange capacity. As hydronium ions interact with the carboxylic acid group more strongly than any other cation, the neutralised negative barrier can be overcome by analyte anions and they can readily interact with quaternary ammonium functional groups (Fig. 3-3-9d).

To compare this effect for the two carboxybetaine surfactants, two separations were obtained using a 10 m*M* $MgCl_2$ eluent, pH 6.4 on DDMAU (Fig. 3-3-10a) and DDMAB (Fig. 3-3-10b) modified particle packed RP columns. The DDMAU coated column exhibited better efficiency and resolution compared to the DDMAB column, predominantly to its higher overall column capacity. The efficiency, resolution and capacity factor values for DDMAU and DDMAB coated columns are presented in Table 3-3-1 and compared to earlier obtained results by O'Riordain *et al.* [8] on a *N*-(dodecyl-*N*,*N*-dimethylammonio)acetate coated stationary phase. It was found that for the DDMAU coated column the efficiency was more than four times greater (~ 12000 – 49000 *N*/m) than for the DDMAB coated column (~2000 – 13000 *N*/m), with improved resolution values for most anion pairs. Comparing these data to results obtained for the DDMAA coated RP column [63], DDMAU and DDMAB columns showed higher selectivity factors for all analytes, while DDMAU showed very high selectivity for iodide and thiocyanate anions.

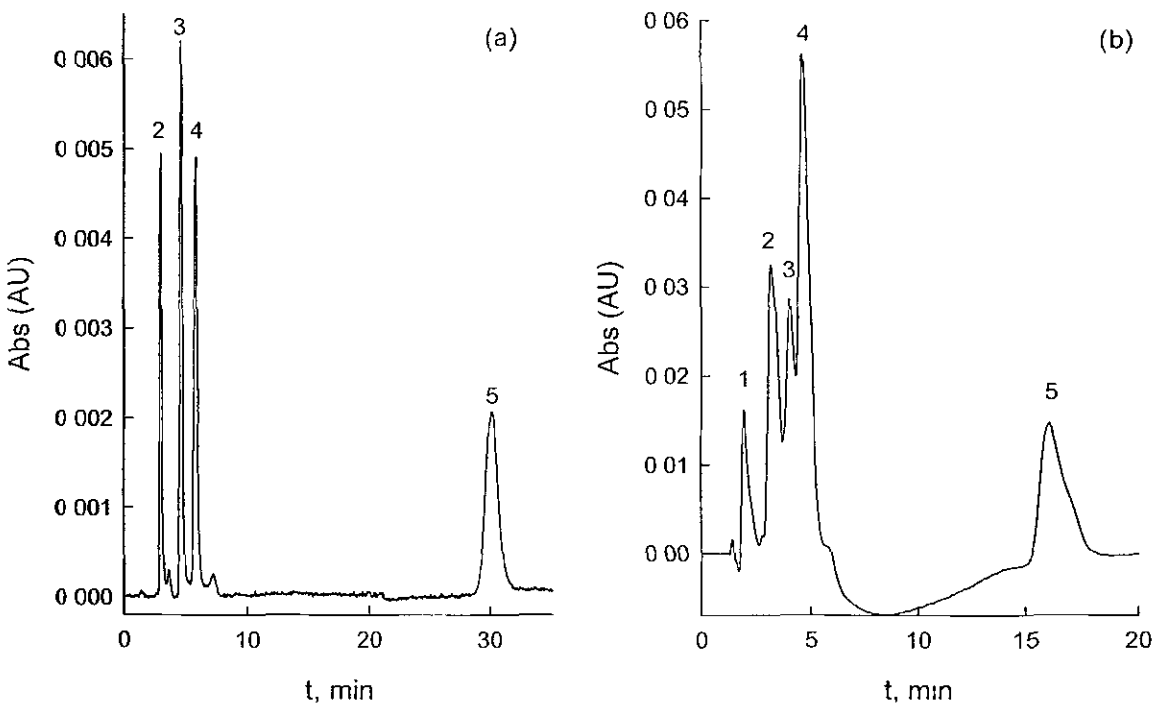


Figure 3-3-10. Separation of a standard mixture of anions. Elution order: 1 – bromate, 2 – nitrite, 3 – bromide, 4 – nitrate, 5 – iodide Column: Gemini C_{18}, 50 x 4.6 mm I.D. coated with DDMAU (a) and DDMAB (b). Eluent. 10 mM $MgCl_2$, pH 6 4, 1.0 mL/min. Direct UV detection at 210 nm

Table 3-3-1. Chromatographic characteristics of N-(dodecyl-N,N-dimethylammonio)alcanoate coated columns (n = 3)

Ion	DDMAU				DDMAB				DDMAA* [8]	
	k	N theor.plates/ column	R_s	α	k	N theor.plates/ column	R_s	α	k	α
iodate	1.03±0.05	196±6	-	-	0.59±0.03	112±17	-	-	-	-
bromate	1.22±0.07	548±5	0.33±0.12	1.12±0.05	0.70±0.05	94±11	0.15±0.03	1.18±0.14	-	-
nitrite	3.02±0.15	733±41	3.05±0.22	2.50±0.16	1.41±0.21	158±18	1.06±0.44	1.99±0.13	0.35	-
bromide	4.00±0.29	762±80	1.33±0.18	1.32±0.04	4.82±0.28	1194±86	5.01±0.37	3.46±0.19	0.57	1.62
nitrate	6.22±0.14	598±36	1.92±0.10	1.58±0.07	8.16±0.19	277±27	1.53±0.13	1.69±0.13	0.84	1.47
iodide	50.2±2.2	2651±172	11.0±0.40	8.34±0.24	17.43±2.0	732±90	3.40±0.35	2.14±0.22	6.20	7.39
thiocyanate	185 0±6.1	2936±155	9.82±0.32	3.69±0.07	49.89±4.1	350±19	2.98±0.12	2.86±0.22	-	-

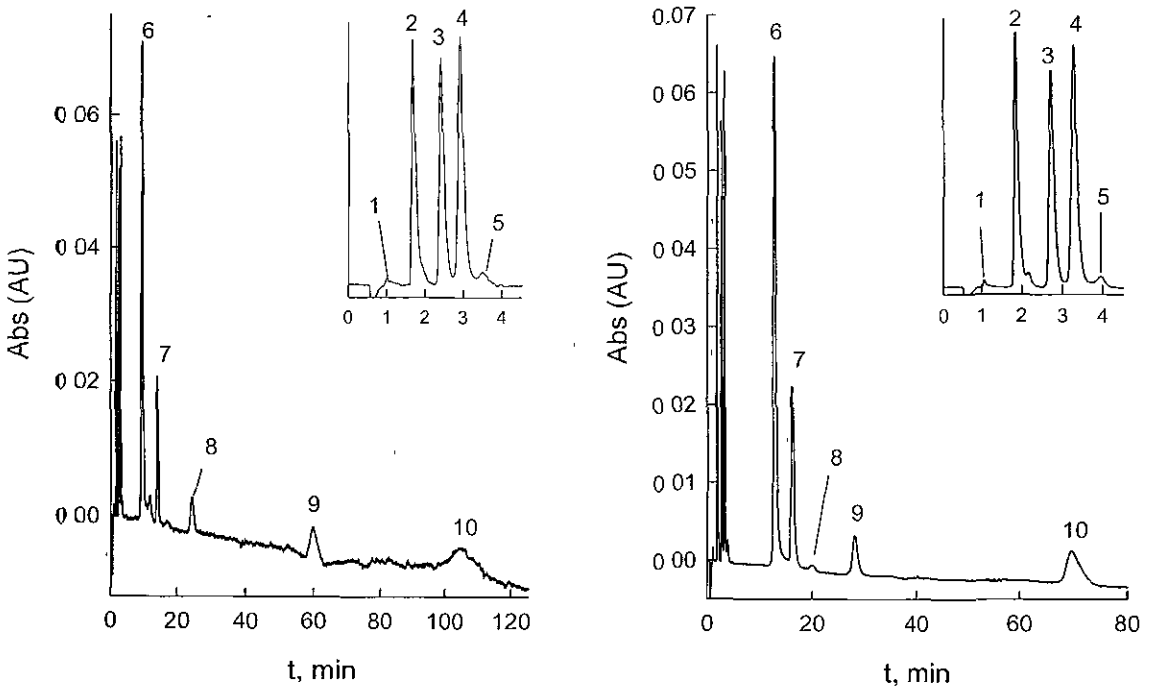
* *N*-(dodecyl-*N*,*N*-dimethylammonio)acetate coated Supelcosil C_{18} column

3.4. Cation charge gradient concept

An obvious way to reduce analysis time is the application of gradient elution using one or more eluent variables. More traditional methods alter some component concentration of the eluent per unit time. It has been reported previously that the use of a cation concentration gradient for the separation of anions on a RP substrate with attached macrocycles has been applied. As the attached macrocycles can complex eluent cations thus generating new anion exchange sites, gradient separations of anions can be performed due to the change of column capacity during the separation achieved by changing the eluent cation [63].

Here a very similar result can be achieved. As the retention times decrease with a decrease in cation charge, it was decided to investigate a "cation charge gradient" for the separation of anions. For the purpose of concept illustration, magnesium and sodium chloride based eluents were selected, as they do not absorb in the UV spectral range. To prove the concept, two separations (with and without cation charge gradient applied) were obtained, whilst keeping the nature and concentration of the eluting anion constant. Without such a gradient applied, and using only 10 m*M* $MgCl_2$ as an eluent, separation of ten test anions on particle packed RP column coated with DDMAU was achieved in an excessive 120 min, as it can be seen in Fig. 3-4-1a. The same mixture was then run with a cation gradient from 10 m*M* $MgCl_2$ to 20 m*M* NaCl for 10 minutes and maintained at 20 m*M* NaCl for the next 70 minutes. Different concentrations of salts were taken in order to keep the similar ionic strength ($I = 2 \times 10^{-4}$ mol/L for NaCl solution and $I = 3 \times 10^{-4}$ mol/L for $MgCl_2$ solution) and exclude its influence. When the gradient profile was applied, the run time was roughly halved (Fig. 3-4-1b). Furthermore, peak shape and baseline resolution of some peaks improved markedly. Visually resolution improved between bromide, nitrate, thiosulphate, dichloroacetate and iodide peaks. Peak asymmetry values for the separation without the gradient are presented in Table 3-4-1. The peak asymmetry factors were calculated at 10 percent of peak height.

An attempt to use cerium salts for the cation charge gradient for the better separation of weakly retained anions was not successful. This was due to the absorption of $CeCl_3$ compounds in the UV spectral range, and therefore once the eluent was switched from $CeCl_3$ to NaCl, an abrupt decrease in the baseline was observed.



(a) *(b)*

Figure 3-4-1. Separation of a standard mixture of anions without (a) and with (b) cation charge gradient applied. Elution order. 1 - acetate, 2 - nitrite, 3 - bromide, 4 - nitrate, 5 - thiosulphate, 6 - dichloroacetate, 7 - iodide, 8 - phthalate, 9 - benzoate, 10 - thiocyanate. Eluent: 10 mM $MgCl_2$ (a) and 10 mM $MgCl_2$ to 20 mM NaCl for 10 min and maintained 20 mM NaCl for next 70 min (b). Column: Gemini C_{18}, coated with DDMAU, flow rate 1.0 mL/min, UV detection at 210 nm

Table 3-4-1. Peaks asymmetries for separations on DDMAU coated column with and without cation gradient

Anion	Peak asymmetry; no gradient	Peak asymmetry; cation gradient	Anion	Peak asymmetry; no gradient	Peak asymmetry; cation gradient
acetate	6.25	3.24	dichloroacetate	2.50	1.0
nitrite	3.70	1.67	iodide	1.29	1.25
bromide	1.67	1.53	phthalate	1.30	1.0
nitrate	2.00	1 80	benzoate	1.50	1.50
thiosulphate	1.28	1.12	thiocyanate	1.16	1.57

3.5. Conclusions

An investigation into novel zwitterionic ion-exchangers prepared by the dynamic coating of particle packed (Gemini C_{18}) and monolithic (Chromolith) RP columns with the carboxybetaine type surfactants, DDMAU and DDMAB has been carried out. The adsorption of surfactants was investigated and possible structures of adsorbed layers were proposed. It was found that coating type and column capacity were dependent on pH and ionic strength as these factors had the strongest affects upon surfactant micelle and internal salt formation The effect of the eluent cation charge on anion retention was also studied. It was shown that the model for the separation mechanism developed for sulphobetaine type surfactants was also applicable to carboxybetaine type phases. A new cation charge gradient elution concept was proposed and its application demonstrated for the reduction of separation time by almost 50 % for a mixture of test anions.

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CHAPTER 4. SEPARATION OF INORGANIC AND ORGANIC ANIONS USING COLUMNS MODIFIED WITH ZWITTERIONIC/AMPHOTERIC SURFACTANTS

Since the first publication on the application of zwitterionic stationary phases to the separation of inorganic anions [1], considerable attention has been attracted to the development of new zwitterionic stationary phases [2-8]. As reported in Chapter 1, the most studied zwitterionic stationary phases up to-date are ODS modified with betaine-type zwitterionic surfactants. Though the most commonly used modifiers have been sulphobetaine type surfactants, recently in a number of works carboxybetaine type surfactants have been utilised [9-11]. Carboxybetaine type surfactants have been mainly used for the separation of inorganic anions. Thus, Hu *et al.* [9] investigated a carboxybetaine type zwitterionic reagent with a rather complex structure, namely 3-(heptadecafluorooctylsulphonylamino)-*N*,*N*-dimethylpropaneammonioethyl-carboxylate ($C_8F_{17}SO_2NHC_3H_6N^+(CH_3)_2C_2H_4COO^-$), which contained a strong inner quaternary ammonium group and a weak terminal carboxyl acid group. The stationary phase coated with this surfactant was applied to the separation of inorganic anions. It was found that when using this stationary phase and eluents at ~ pH 8.5, no retention of anions was observed, but once switched to acidic eluent a strong retention of inorganic anions was observed. However, this surfactant was not ideal for ZIC as it contained an additional polar sulphonylamido group, which could affect the ion-exchange properties of the resultant stationary phase. More recently O'Riordain *et al.* [10,11] investigated (dodecyldimethyammonio)acetic acid as a column modifier, which also contained a strong inner quaternary ammonium group and a weak terminal carboxylic acid group. Particle packed and monolithic RP columns were applied for the separation of inorganic anions. It was found that selectivity was also dependent on the pH and ionic strength of the eluent. With an increase in pH a decrease in retention for anions was observed, while with a decrease in pH, all anions were strongly retained. For all previously studied carboxybetaine type surfactants it was found that these stationary phases exhibit strong affinity to iodide, thiocyanate and perchlorate.

The aim of Chapter 4 was the study of anion exchange properties of monolithic and particle packed RP columns modified with *N*-(dodecyl-*N*,*N*-dimethylammonio)alcanoates with different intercharge chain length, in relation to eluent concentration and pH. Anion separations were obtained in optimised conditions.

Phosphate buffer solutions were chosen as the eluent due to its compatibility with a UV-absorbance detection at 210 nm.

4.1. Effect of eluent concentration on the retention of anions

The effect of eluent concentration was studied for RP C_{18} monolithic (Chromolith) columns, coated with DDMAU and DDMAB and for RP C_{18} particle packed columns (Gemini), coated with the same surfactants. In Section 3.3 2 , the effect of eluent concentration has already been discussed in relation to the retention mechanism of anions. In the current section it is investigated for separation optimisation.

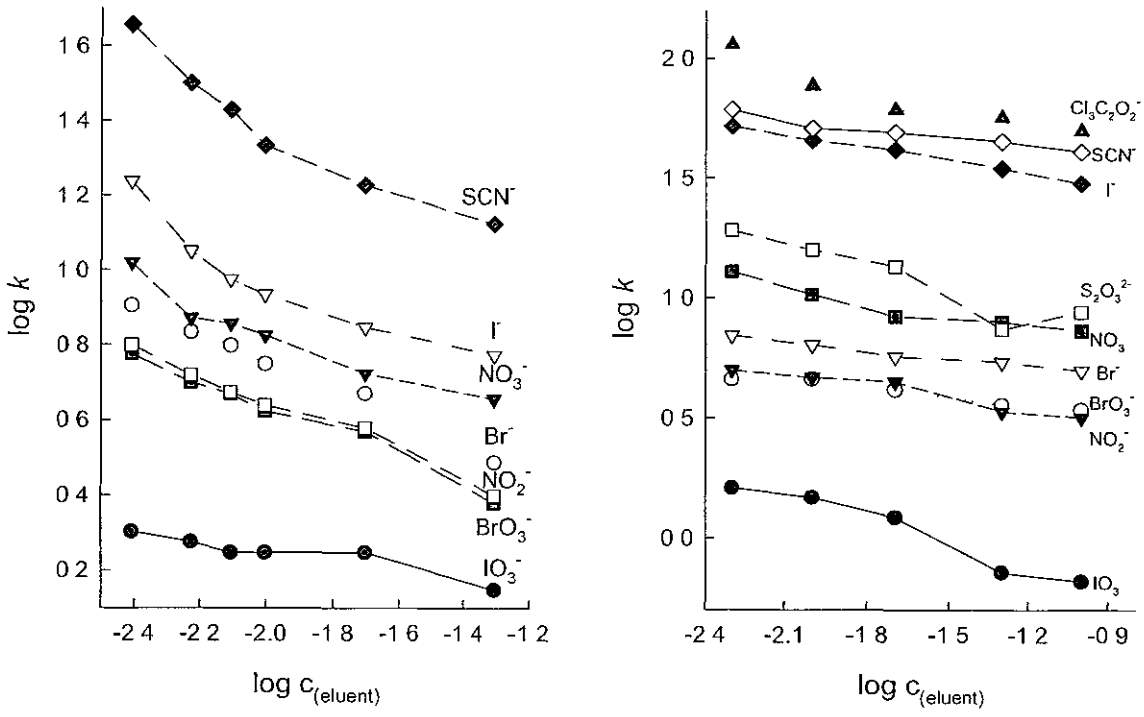
4.1 1 Particle packed RP columns coated with DDMAU and DDMAB

For the effect of eluent concentration on the retention of iodate, bromate, nitrite, bromide, nitrate, iodide, thiosulphate, thiocyanate and trichloroacetate, a series of eluents of different concentrations, ranging from 4 to 50 m*M*, each adjusted to pH 5.2 were made. For the study of the DDMAB modified column, eluents were prepared in 2 m*M* DDMAB solution in order to stabilise the coating. The effect of altering the eluent concentration for particle packed columns coated with DDMAB and DDMAU is shown in Fig. 4-1-1. For both surfactants the concentration dependences exhibited linear character. A decrease in retention was observed with an increase in eluent concentration showing that the solute-sorbent interaction displayed anion exchange properties.

The slope for each plot was less than expected from a simple ion-exchange mechanism (Table 4-1-1), where the slope of the resulting plot should equal the value calculated from dividing the charge of the analyte anion by the charge of eluent anion [12] as in eq. 4-1-1:

$$\log k = const - \frac{z_A}{z_e} \log c_e \qquad \text{(eq. 4-1-1)},$$

where the *const* is the equilibrium constant for the exchange process; z_A and z_e are charges for analyte and eluent ions respectively; and c_e is eluent concentration. The fact that slopes for all anions were smaller than expected for ion-exchange reflects the zwitterionic nature of both stationary phases at the pH of study.



DDMAB *DDMAU*

Figure 4-1-1. Dependence of retention factor on concentration of eluent (phosphate buffer, mM) for chloride, iodate, bromate, nitrite, bromide, nitrate, thiosulphate, iodide, thiocyanate and trichloroacetate Column. Gemini C_{18}, coated with DDMAB or DDMAU, 50 x 4 6 mm I.D.

Table 4-1-1. The slope values for log k vs log c dependencies for particle packed column coated with DDMAB and DDMAU

Anion	IO_3^-	NO_2^-	BrO_3^-	Br^-	NO_3^-	I^-	SCN^-
Gemini C_{18} coated with DDMAB							
R^2	0.908	0.974	0 973	0.974	0.904	0.920	0.929
slope	-0.335	-0.111	-0.164	-0.109	-0.184	-0.379	-0.465
intercept	-0.527	0.420	0.337	0.585	0.661	0.228	0.466
Gemini C_{18} coated with DDMAU							
R^2	0.949	0.979	0.984	0.992	0.969	0.996	0.944
slope	-0.123	-0.339	-0.342	-0.369	-0.303	-0.179	-0.120
intercept	4.383	-0.046	-0.0349	0.0217	0.232	1.304	1.490

The elution order was found to be the same for both stationary phases:

$$IO_3^- < BrO_3^- \approx NO_2^- < Br^- < NO_3^- < S_2O_3^{2-} < I^- < SCN^- < Cl_3C_2O_2^-.$$

and this order did not change with increasing eluent concentration. Lower capacity factor values for the column coated with DDMAB can be explained by a lower coating stability compared to DDMAU. Greater selectivity and improved coating stability makes DDMAU the more preferable surfactant for coating modification. At the eluent pH 5.2 the optimised phosphate buffer concentration was found to be 10 m*M* for both stationary phases.

4 1.2. Monolithic RP columns coated with DDMAU and DDMAB

The same experiments were run for monolithic RP columns coated with DDMAB and DDMAU. For the effect of eluent concentration on the retention of iodate, bromate, nitrite, bromide, nitrate, iodide, thiosulphate, thiocyanate and trichloroacetate, a series of eluents of different concentrations ranging from 5 to 100 m*M*, each adjusted to pH 5.2 were prepared. For the study of DDMAB modified column, eluents were prepared in 2 m*M* DDMAU solution in order to stabilise the coating. The effect of altering the eluent concentration for the monolithic RP columns coated with different surfactants is shown in Fig 4-1-2. For both stationary phases the concentration dependencies exhibited linear character, similar to those observed for particle packed RP based stationary phases. A decrease in retention with an increase in eluent concentration shows that the solute-sorbent interaction displayed anion exchange properties, with the reduction of retention factor values a result of the increase of the eluent strength with the increase in its concentration. A slight difference in the observed dependencies was seen between particle packed RP and monolithic RP columns coated with DDMAB. In the first instance, slopes for dependencies for all anions were less compared to those performed on the monolithic RP column modified with DDMAB (Table 4-1-2.). The possible reason for this might be not complete coating of monolithic silica column with first, ODS and second, with surfactant and as a result additional interactions with non-modified silanol groups could be present. However, as was the case for the particle packed column, the slopes for all anions for both zwitterionic coatings were, as expected, less than expected for anion exchange dependence and reflected the zwitterionic nature of stationary phases at the current pH.

The elution order (higher selectivity to bromate and nitrite) was also slightly different from the one observed for the Gemini C_{18} – based stationary phases, but was the same for both DDMAU and DDMAB coated Chromolith columns:

$$IO_3^- < NO_2^- < BrO_3^- < Br^- < NO_3^- < S_2O_3^{2-} < I^- < SCN^- < Cl_3C_2O_2^-.$$

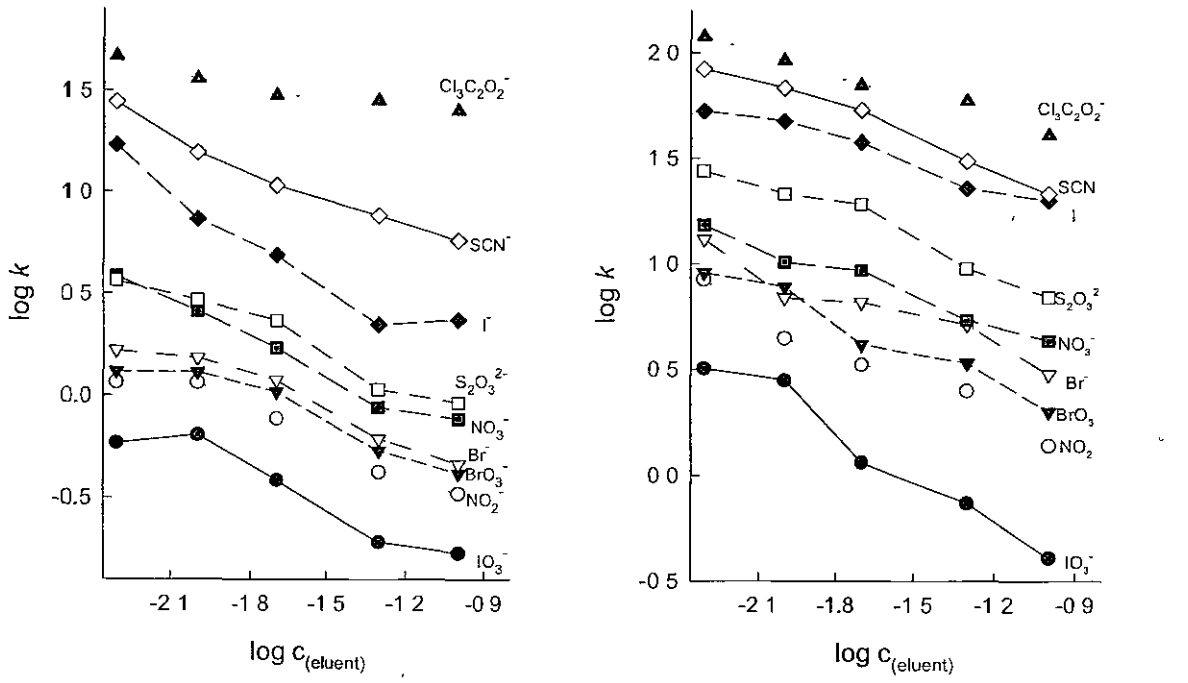


Figure 4-1-2. Dependence of retention factor on concentration of eluent (phosphate buffer, mM) for chloride, iodate, bromate, nitrite, bromide, nitrate, thiosulphate, iodide, thiocyanate and trichloroacetate. Column. Chromolith RP, coated with DDMAB or DDMAU, 100 x 4.6mm I D.

Table 4-1-2. The slope values for log k vs log c dependencies for monolithic RP coated with DDMAB and DDMAU

Anion	IO_3^-	NO_2^-	BrO_3^-	Br^-	NO_3^-	I^-	SCN^-
Chromolith RP coated with DDMAB							
R^2	0.914	0.950	0.924	0.953	0.981	0.923	0.969
slope	-0.491	-0.466	-0.423	-0.461	-0.567	-0.676	-0.504
intercept	-1.279	-0.942	-0.783	-0.779	-0.729	-0.422	0.225
Chromolith RP coated with DDMAU							
R^2	0.967	0.963	0.962	0.914	0.979	0.967	0.982
slope	-0.717	-0.547	-0.505	-0.423	-0.417	-0.359	-0.468
intercept	-1.091	-0.381	-0.179	0.089	0.212	0.928	0.882

This observed elution order did not change with an increase in eluent concentration. Lower capacity factor values for the column coated with DDMAB again resulted from a lower coating stability comparing to DDMAU. As with the particle packed columns, DDMAU was preferable for modification due to this improved stability and the

resultant capacity. At an eluent pH of 5.2 the optimised phosphate buffer concentration was found to be 50 m*M* for both stationary phases.

4.2. Effect of eluent pH on the retention of anions

The pH of the eluent strongly affected the retention of anions on both zwitterionic surfactants, which has also been shown previously with similar carboxybetaine type surfactants [10] As the DDMAU and DDMAB molecules contain weak carboxylic acid terminal groups, a reduction in pH reduces the number of dissociated groups on the surface and hence effectively increases anion exchange capacity. The effect of eluent pH was investigated for both particle packed Gemini C_{18} and monolithic Chromolith RP columns coated with DDMAB and DDMAU using the optimised eluent concentration of 10 m*M* phosphate buffer over a pH interval 3.7 to 6.5. For the study of stationary phases coated with DDMAB, eluents were once again prepared in 2 m*M* DDMAB in order to stabilise surfactant coating.

4 2.1.Particle packed RP columns coated with DDMAU and DDMAB

From Fig. 4-2-1 it can be observed that there was a noticeable increase in retention for all anions studied on both stationary phases with a reduction in eluent pH.

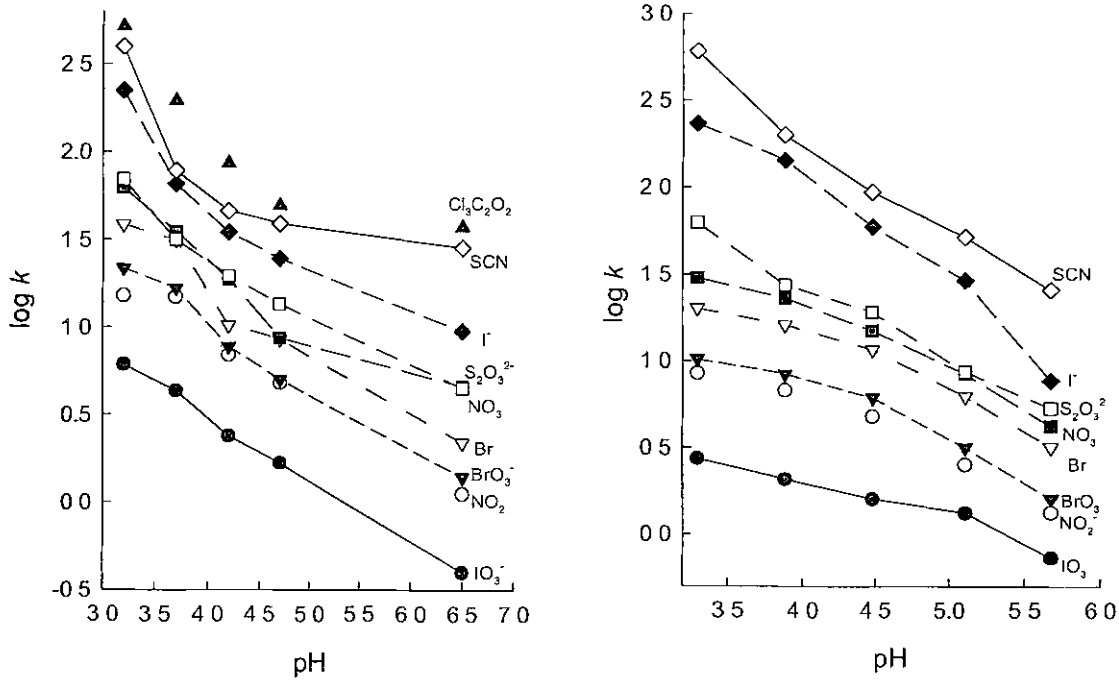


Figure 4-2-1. Dependence of retention factor for iodate, bromate, nitrite, bromide, nitrate, thiosulphate, iodide, thiocyanate and trichloroacetate on eluent pH. Column: Gemini C_{18}, coated with DDMAB or DDMAU, 50 x 4.6 mm I D.

There was an indication of more rapid increase in retention observed between pH 3.2 and 3.8 for iodide, thiocyanate and thiosulphate for the DDMAU modified stationary phase and for iodide, thiocyanate and trichloroacetate for the DDMAB-coated phase. The decrease in the retention of anions with an increase in pH occurs due to the increase of the negative charge on the surface of the phase (as described in Section 3.3.3). Also with the increase in pH the elution power of phosphate eluent increases resulting in decrease of anion retention The elution order was the same and did not change for all anions investigated on both stationary phases:

$$IO_3^- < NO_2^- < BrO_3^- < Br^- < NO_3^- < S_2O_3^- < I^- < SCN^- < Cl_3C_2O_2^-.$$

The optimal pH for separation was found to be 4.6 for the column coated with DDMAB and 4.5 for DDMAU coated phase. Resolution on both columns markedly increased at lower pH (Fig. 4-2-2), highlighting that the selectivity of the column could be manipulated for selected anions through adjustment of eluent pH and could be used to maximise both resolution and overall analysis time.

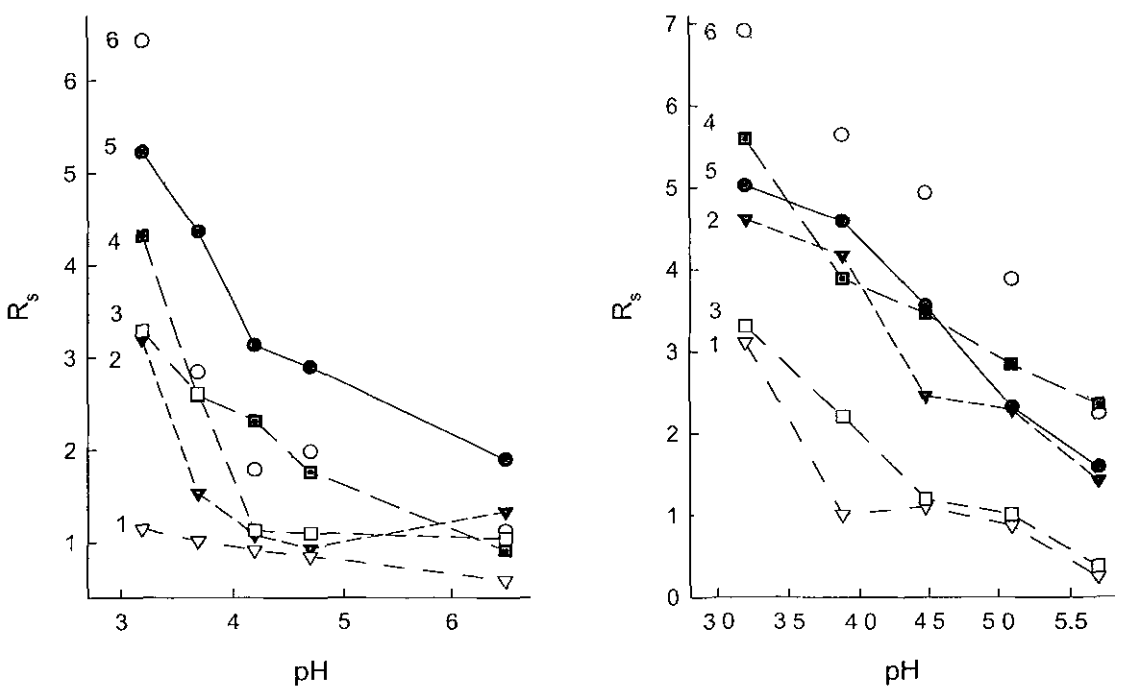


Figure 4-2-2. Peak resolution for selected peak pairs as a function of eluent pH. 1 – NO_2^-/IO_3^-, 2 – NO_3^-/Br^-, 3 – SCN^-/I^-, 4 – I^-/NO_3^-, 5 – BrO_3^-/NO_2^-, 6 – Br^-/BrO_3^-. Column: Gemini C_{18}, coated with DDMAB or DDMAU, 50 x 4.6 mm I.D. Resolution calculated as. $R_s = 2\left(\frac{t_{R2} - t_{R1}}{w_1 + w_2}\right)$

The observed effect was more significant for the stationary phase coated with DDMAB.

Table 4-2-1. Selectivity and efficiency data for a range of inorganic anions on particle packed RP columns coated with DDMAB and DDMAU. Eluent: 10 mM phosphate buffer, pH range 3.2 to 6.5. Efficiency was calculated according to: $N = 5.54 \times \left(\frac{t_R}{w_{1/2}} \right)^2$ *for the column length (50 mm)*

Gemini C_{18} coated with DDMAB													
	IO_3^-	NO_2^-		BrO_3^-		Br^-		NO_3^-		I^-		SCN^-	
pH	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*
3.2	966	3.45	527	1.43	1633	2.52	1890	1.82	1152	3.55	582	1.62	449
3.7	1191	3.75	618	1.12	969	2.09	498	1.42	412	1.27	347	1.48	446
4 2	951	3.00	423	1.11	520	1.22	373	1.23	234	1.28	159	1.28	427
4.7	1086	2.50	404	1.16	262	2.08	269	1.17	225	1.60	449	1.13	385
6.5	299	1.80	278	1.03	170	1.86	162	1.09	151	2.03	260	2.95	261
Gemini C_{18} coated with DDMAU													
	IO_3^-	NO_2^-		BrO_3^-		Br^-		NO_3^-		I^-		SCN^-	
pH	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*
3.2	948.74	2.05	771	2.90	1048	2.17	2745	1.49	3068	28.71	738	2.46	298
3.9	694.30	1.93	526	2.85	913	1.87	2511	1.41	4185	8.55	312	1.40	449
4.5	537.42	1.75	351	2.66	621	1.82	2072	1.27	2246	6.10	276	1.58	171
5.1	328.95	1.31	692	2.33	357	1.84	1338	1.32	1580	5.82	594	1.08	400
5.7	242.08	1.15	388	1.88	294	1.74	567	1.28	834	2.89	222	1.03	290

Table 4-2-1 shows the selectivity and efficiency data for a range of UV-absorbing anions on Gemini C_{18} columns coated with DDMAB and DDMAU. It was shown that with an increase in pH, both selectivity and efficiency increased for the majority of anions. Selectivities were similar for both stationary phases, which showed strong retention for polarisable anions, such as iodide and thiocyanate. Both columns exhibited good selectivity for a number of peak pairs including bromate/bromide, iodate/iodide and nitrite/nitrate. The efficiency for the majority of anions was higher for the DDMAU coated column.

4.2.2 Monolithic RP columns coated with DDMAU and DDMAB

For the investigation of the effect of eluent pH on the retention of anions on monolithic RP columns coated with different surfactants a series of phosphate buffer eluents with optimised concentration of 10 m*M* and pH between 3.2 and 6.0 were used. For the study of the effect on DDMAB modified column, eluents were prepared in 2 m*M* DDMAB solution. As shown in Fig. 4-2-3, the same effect of increasing retention for all anions on both stationary phases with a reduction in eluent pH was observed.

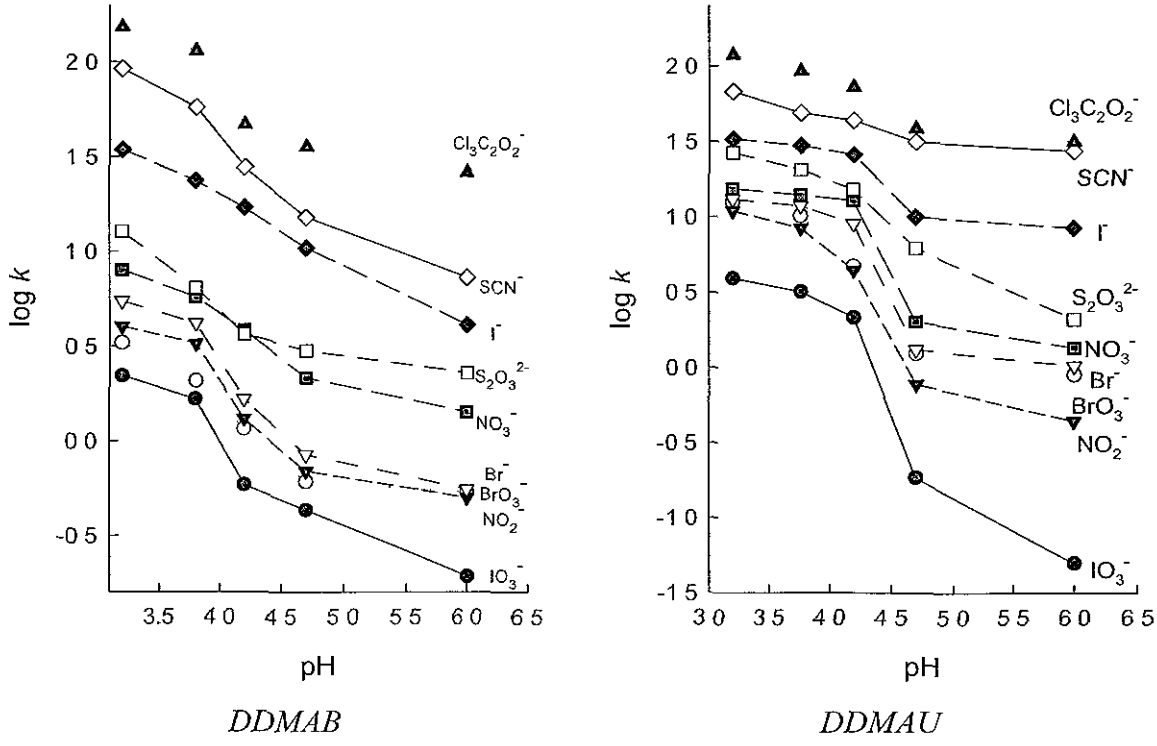


Figure 4-2-3 Dependence of retention factor for iodate, bromate, nitrite, bromide, nitrate, thiosulphate, iodide, thiocyanate and trichloroacetate on eluent pH. Column. Chromolith RP, coated with DDMAB or DDMAU, 100 x 4.6 mm I.D.

Unlike the particle packed RP stationary phases coated with DDMAU and DDMAB, no significant rapid increase in retention was observed between pH 3.2 and 3.8 for iodide, thiocyanate and thiosulphate and trichloroacetate. In fact, a more rapid increase was present between pH 3.8 and 4.6 for the DDMAB-coated column, and between pH 4.2 and 4 6 for the DDMAU-coated column for all anions of interest. The total increase in retention for all anions was less than that observed for both DDMAB and DDMAU modified particle packed RP stationary phases. In addition, it can be seen that strongly retained anions such as iodide, thiocyanate and trichloroacetate were less retained on the monolithic RP columns coated with both surfactants compared to the particle packed columns.

The elution order observed was the same as that seen with the particle packed RP columns and did not change for all anions investigated on both stationary phases:

$$IO_3^- < NO_2^- < BrO_3^- < Br^- < NO_3^- < S_2O_3^- < I^- < SCN^- < Cl_3C_2O_2^-.$$

The optimal pH for separation was found to be pH 4.6 for both columns although according to Fig.4-2-3, selectivity was higher at pH 6.0.

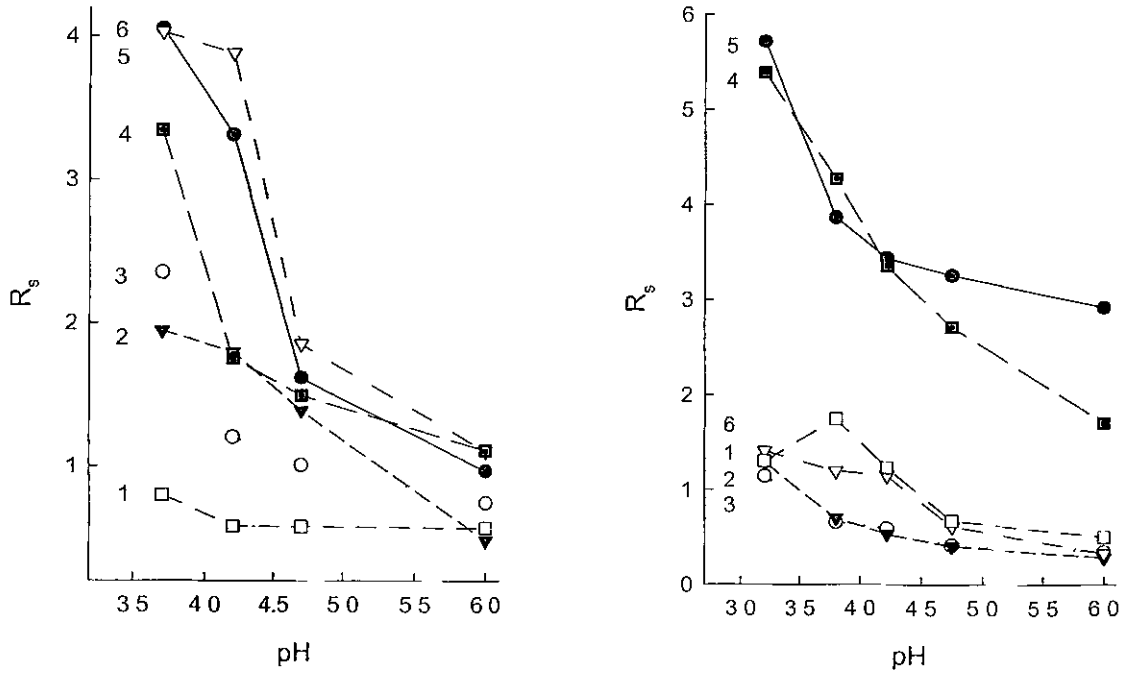


Figure 4-2-4 Peak resolution for selected peak pairs as a function of eluent pH 1 – SCN^-/I^-, 2 – NO_3^-/Br^-, 3 – Br^-/BrO_3^-, 4 – I^-/NO_3^-, 5 – BrO_3^-/NO_2^-, 6 – NO_2^-/IO_3^-. Column: Chromolith RP, coated with DDMAB or DDMAU, 100 x 4 6 mm I.D. Resolution calculated as: $R_s = 2\left(\frac{t_{R2} - t_{R1}}{w_1 + w_2}\right)$

However, as can be seen from Table 4-2-2, the efficiency was relatively poor compared to that at lower pH values. The resolution for both columns markedly increased at lower pH, as it can be seen in Fig. 4-2-4, highlighting that the selectivity of the column could be manipulated for selected anions through adjustment of eluent pH and could be used to maximise both resolution and overall analysis time. This effect was more pronounced for the particle packed RP columns modified with both surfactants, with the increase in resolution less clear for the monolithic RP columns. The observed effect was stronger for the stationary phases coated with DDMAB.

Table 4-2-2 shows the selectivity and efficiency data for a range of UV-absorbing anions on monolithic RP columns coated with DDMAB and DDMAU. It is shown that with an increase in pH, both selectivity and efficiency increased for the majority of anions (except iodide and thiocyanate). Selectivities were similar for both stationary phases and showed strong retention for polarisable anions, such as iodide and thiocyanate, though the DDMAU coated column showed stronger affinity for iodide. Both columns exhibited good selectivity for a number of anion pairs including bromate/bromide, iodate/iodide and nitrite/nitrate. The efficiency for the majority of anions was higher for DDMAU coated column.

Table 4-2-2. Selectivity and efficiency data for a range of inorganic anions on monolithic RP columns coated with DDMAB and DDMAU. Eluent: 10 mM phosphate buffer, pH range 3.7 to 6.0. Efficiency was calculated according to: $N = 5.54 \times \left(\frac{t_R}{w_{1/2}} \right)^2$ *for the column length (100 mm)*

Chromolith RP coated with DDMAB													
	IO_3^-	NO_2^-		BrO_3^-		Br^-		NO_3^-		I^-		SCN^-	
pH	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*
3.7	467	1.57	789	2.20	1200	1.95	425	1.68	398	2.69	229	1.72	159
4.2	506	1.35	955	1.99	997	1.24	299	1.42	408	1.41	473	2.43	116
4.7	487	2.35	960	1.66	490	0.78	432	0.99	401	4.32	342	2.50	116
6.0	298	2.64	902	1.42	337	0.51	488	0.82	361	5.34	134	2.65	115
Chromolith RP coated with DDMAU													
	IO_3^-	NO_2^-		BrO_3^-		Br^-		NO_3^-		I^-		SCN^-	
pH	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*	α	*N*
3.2	223.00	1.14	1112	1.04	1692	2.56	513	1.07	336	7.91	162	1.20	85
3.9	203.90	1.23	988	1.16	1699	2.39	273	1.41	340	6.27	168	1.23	209
4.5	236.04	1.29	277	1.19	1930	1.42	118	1.20	110	2.23	622	4.34	199
5.1	221.60	0.81	309	1.51	798	2.51	678	1.12	527	4.09	554	3.18	107
5.7	197.91	0.69	484	1.58	738	2.41	398	1.20	419	4.70	824	1.38	346

4.3. Separation of anions

It was shown earlier that all stationary phases of interest, including both monolithic and particle packed RP columns, each coated with either DDMAU or DDMAB, could all be potentially used for the separation of anionic species. As was discussed in Sections 4.1 and 4.2, pH and concentration of the eluent both have a strong effect on the retention of anions, Therefore a systematic approach to separation and optimisation was applied here.

4 3.1. Optimisation of anion separations

For optimisation of separations the approach suggested by Ng *et al.* was applied [13]. For optimisation, an experimental surface was designed, governed by the eluent pH and ionic strength. Nine individual experiments were run within the pH range between pH 3.0 and 6.0, and concentration range between 10 m*M* and 100 m*M*. The resulting chromatograms were assessed using the resolution product criterion, *r*, calculated according to eq. 4-3-1.

$$r = \prod_{i=1}^{n-1} \left(\frac{R_{S(i,i+1)}}{\frac{1}{n-1} \sum_{i=1}^{n-1} R_{S(i,i+1)}} \right) \qquad \text{(eq. 4-3-1)}$$

where n is number of peaks, $R_{S(i,i+1)}$ is the resolution for the adjacent peaks i and $i+1$ and calculated according to eq.4-3-2:

$$R_{S(i,i+1)} = 2\left(\frac{t_{Ri+1} - t_{Ri}}{w_{i+1} + w_i} \right) \qquad \text{(eq. 4-3-2)}$$

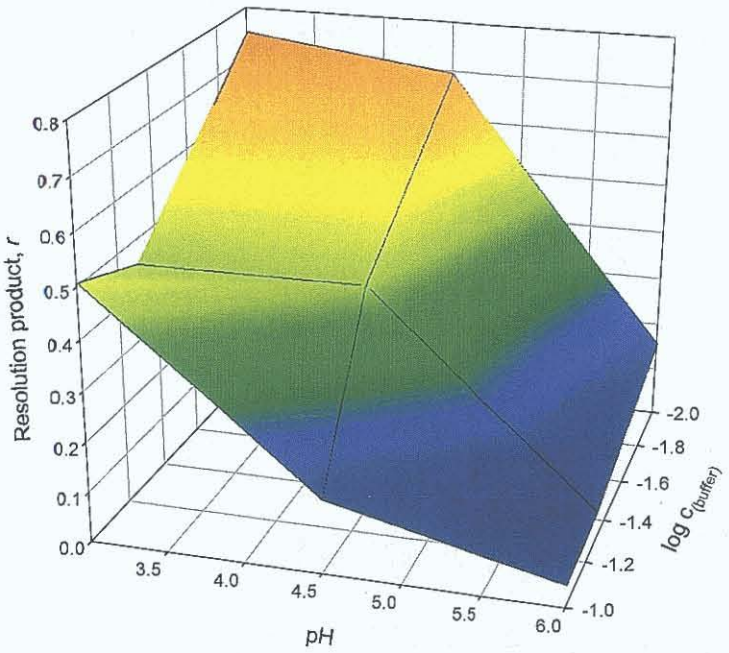
where t_R is retention time and w is peak width.

The resolution product r has values in the range of $0 < r < 1$. A value of 1 indicates that all R_S values are equal, *i.e.* when all peaks are distributed evenly over the chromatogram and conversely a value of zero indicates co-elution of two or more peaks somewhere within the chromatogram. The optimum eluent composition is given by the highest value of the resolution product criterion over the experimental surface.

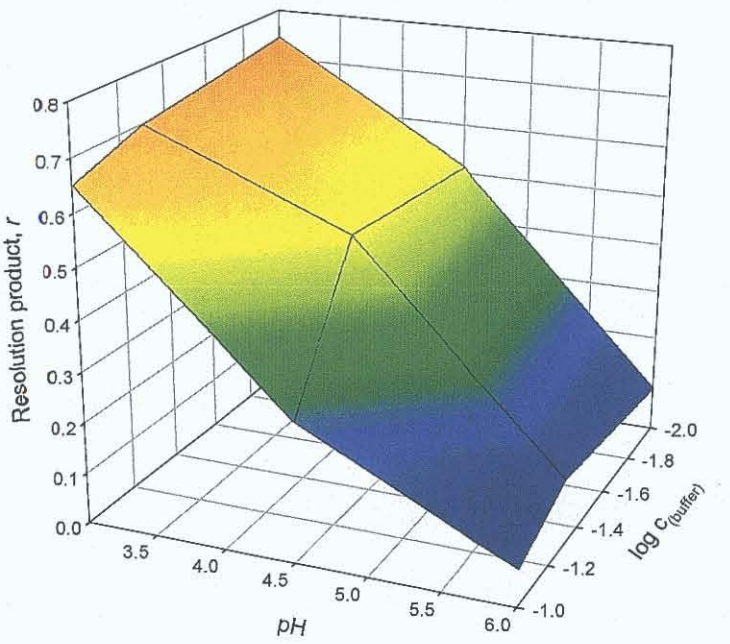
4.3.1.1. Particle packed RP columns coated with DDMAU and DDMAB

For the experiment nine eluents were prepared: 10 m*M* phosphate buffer, pH 3.0, 4.5 and 6.0; 50 m*M* phosphate buffer, pH 3 0, 4.5 and 6.0 and 100 m*M* phosphate

buffer, pH 3.0, 4.5 and 6.0. For the study of the column coated with DDMAB, all eluents were prepared in 2 m*M* DDMAB solution in order to stabilise coating. For the calculated r – values, a response surface was constructed, which is shown in Fig. 4-3-1 for both modified phases.



(a) Column: Gemini C_{18} coated with DDMAB, 50 x 4.6 mm I.D.



(b) Column: Gemini C_{18} coated with DDMAU, 50 x 4.6 mm I.D.

Figure 4-3-1. Resolution response surface for eluent pH and concentration optimisation. Standard mixture: iodate, nitrite, bromide, nitrate, iodide, thiocyanate

The optimum combination of the pH and buffer concentration was determined from the apex of the response surface within the experimental space. The response curve permits

the identification of the eluent condition where all analytes were optimally resolved. For the DDMAB coated column it was found to be 10 m*M* phosphate buffer, pH 3.0, and for the column coated with DDMAU it was 50 m*M* phosphate buffer, pH 3.0. The chromatograms corresponding to these conditions are shown in Fig. 4-3-2. While under these suggested conditions all peaks were completely resolved, the overall retention time was still excessively long due to strong retention of iodide and thiocyanate at low pH. The solution of this problem could be the application of pH gradient to this separation as increasing eluent pH results in decreasing anion retention time.

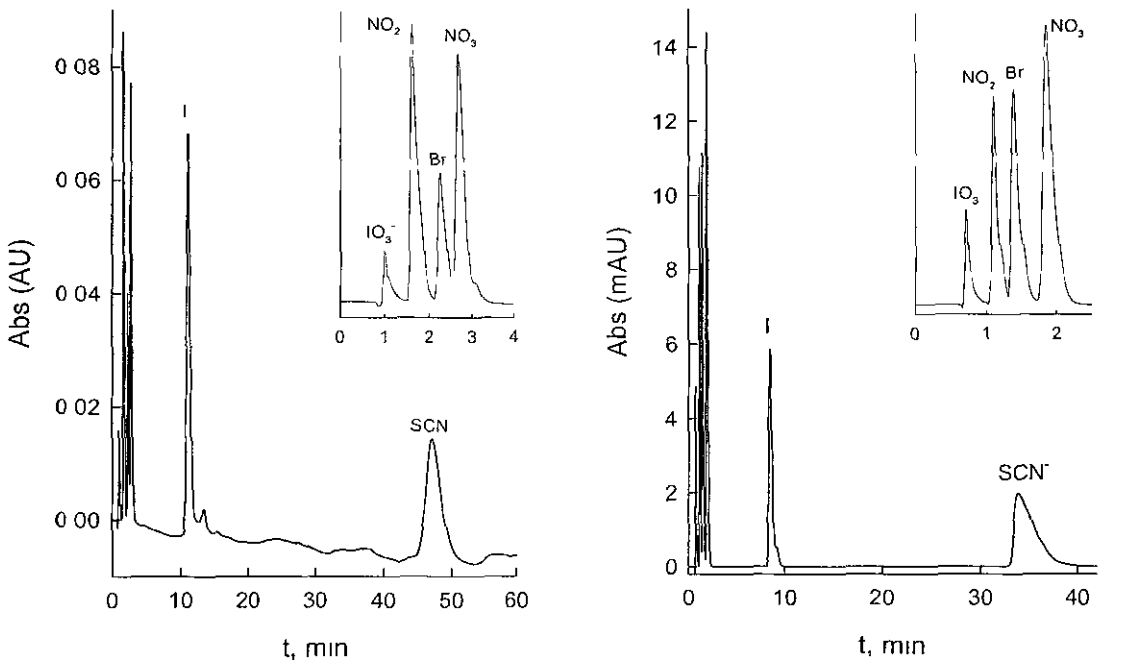


Figure 4-3-2. Chromatogram of standard mixture of six anions: iodate, nitrite, bromide, nitrate, iodide and thiocyanate. (a) Column Gemini C_{18} coated with DDMAB, 50 x 4.6 mm I.D.; Eluent: 10 mM phosphate buffer, 2 mM DDMAB, pH 3.0, (b) Column Gemini C_{18} coated with DDMAU, 50 x 4 6 mm I D.; Eluent. 50 mM phosphate buffer, pH 3.0, F = 1.0 mL/min

Chromatographic parameters for separations on both columns are shown in Table 4-3-1. It can be seen that all peaks on the chromatogram are completely resolved. Stronger anion retention on the column coated with DDMAB was due to the additive of the surfactant to the eluent, as well as a lower eluent concentration being used. It was shown that peak efficiency for the DDMAU coated column was ~ 35000 theoretical plates per meter, compared to 10400 theoretical plates for the DDMAB coated column. The reason for this poorer efficiency may also be related to the eluent concentration of

DDMAB, but in addition could be related to the differences in the intercharge chain length.

Table 4-3-1. Chromatographic parameters for separations of standard anion mixtures

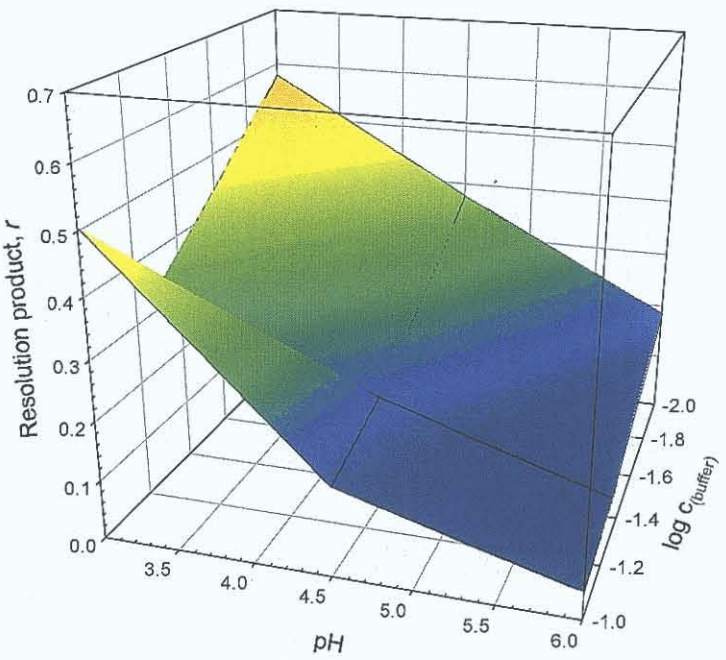
anion	Gemini C_{18} coated with DDMAB				Gemini C_{18} coated with DDMAU			
	k	α	N (per meter)	R_S	k	α	N (per meter)	R_S
IO_3^-	0.72	-	2080	-	0.35	-	4560	-
NO_2^-	3.46	4.82	2600	1.75	1.21	3.45	10400	1.77
Br^-	5.25	1.52	3900	1.01	2.04	1.70	15140	1.60
NO_3^-	8.24	1.57	6460	1.45	2.65	1.30	13280	1.02
I^-	43.82	5.32	7640	3.88	15.81	5.97	34880	9.74
SCN^-	184.16	4.20	10420	4.33	70.07	4.43	19680	11.58

4.3.1.2. Monolithic RP columns coated with DDMAU and DDMAB

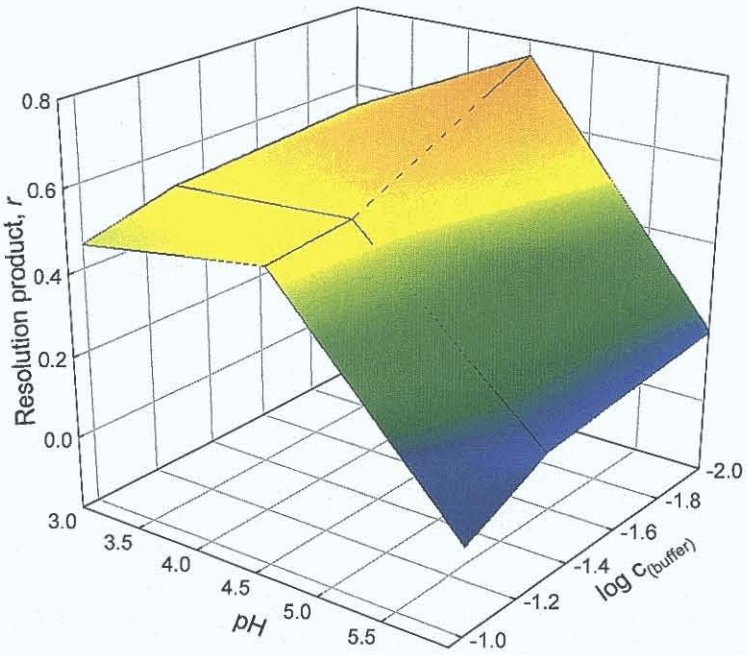
For this experiment, nine eluents were prepared: 10 m*M* phosphate buffer, pH 3.0, 4.5 and 6.0; 50 m*M* phosphate buffer, pH 3.0, 4.5 and 6.0 and 100 m*M* phosphate buffer, pH 3.0, 4.5 and 6.0. For the study of the column coated with DDMAB, eluents were prepared in 2 m*M* DDMAB solution in order to stabilise coating. For the calculated r – values a response surface was constructed. It is shown in Fig. 4-3-3 for both stationary phases. For the monolithic RP coated with DDMAB optimal conditions were found to be 10 m*M* phosphate buffer, pH 3.0, and for the column coated with DDMAU this was 10 m*M* phosphate buffer, pH 4.5. The chromatograms corresponding to these conditions are shown in Fig. 4-3-4. Chromatographic parameters for separations are shown in Table 4-3-2.

Table 4-3-2. Chromatographic parameters for separations of standard anion mixtures

anion	Chromolith RP coated with DDMAB				Chromolith RP coated with DDMAU			
	k	α	N (per meter)	R_S	k	α	N (per meter)	R_S
IO_3^-	0.63	-	8995	-	0.53	-	8975	-
NO_2^-	1.41	2.61	7622	1.13	1.56	2.94	7751	1.59
Br^-	2.05	1.46	9774	0.96	2.11	1.35	2374	1.24
NO_3^-	5.78	2.82	12091	2.99	6.46	3.06	6278	1.11
I^-	27.26	4.71	11668	2.86	10.83	1.68	8087	1.62
SCN^-	102.6	3.78	12465	8.14	62.70	5.70	3131	1.66



(a) Column: Chromolith RP coated with DDMAB, 100 x 4.6 mm I.D.



(b) Column: Chromolith RP coated with DDMAU, 100 x 4.6 mm I.D.

Figure 4-3-3. Resolution response surface for eluent pH and concentration optimization. Standard mixture: iodate, nitrite, bromide, nitrate, iodide, thiocyanate

It can be seen that all peaks on the chromatogram were completely resolved. Again, stronger anion retention on the column coated with DDMAB could be due to the addition of the surfactant to the eluent as well as lower eluent pH being used. Compared to the particle packed RP based columns, the efficiency was found to be higher for the DDMAB monolithic phase, which showed ~ 12500 theoretical plates per meter, while

for the DDMAU coated column this was ~ 9000. However, the overall separation time under optimal conditions was higher for monolithic columns.

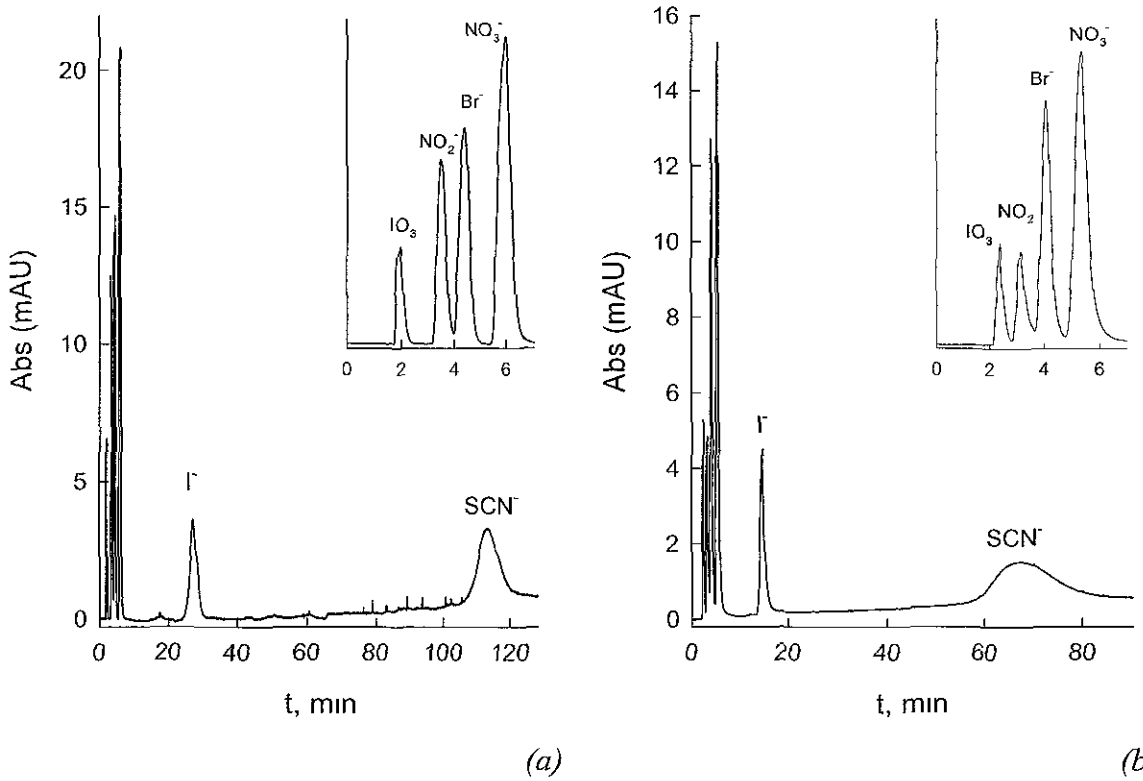


Figure 4-3-4. Chromatogram of standard mixture of six anions. iodate, nitrite, bromide, nitrate, iodide and thiocyanate. (a) Column Chromolith RP coated with DDMAB, 100 x 4.6 mm I.D.; Eluent· 10 mM phosphate buffer, 2 mM DDMAB, pH 3.0, (b) Column Chromolith RP coated with DDMAU, 100 x 4.6 mm I.D ; Eluent: 10 mM phosphate buffer, pH 4.5, F = 1.0 mL/min

4.3 2. Application of a pH gradient to the separation of anions

As was shown in Section 4.3.1., the resolution of all anion peaks can be achieved at low concentration and low pH of the eluent. However, the overall separation time can exceed 140 min, which is unacceptable. As the retention time reduces with increasing eluent pH and concentration, it was decided to apply a pH gradient to the separation of anions. For this investigation a particle packed RP column coated with DDMAU was selected, as in previous studies it produced best efficiency and resolution of the targeted anions.

As can be seen in Fig.4-3-5, increasing eluent pH above 6.0 decreased the retention of iodide and thiocyanate markedly. But at the same time a large system peak and change in baseline was observed on the chromatogram. Further study of the

observed appearance of a system peak is described in Section 4.4. The change in baseline was apparent due to impurities in reagents used for preparation of eluents.

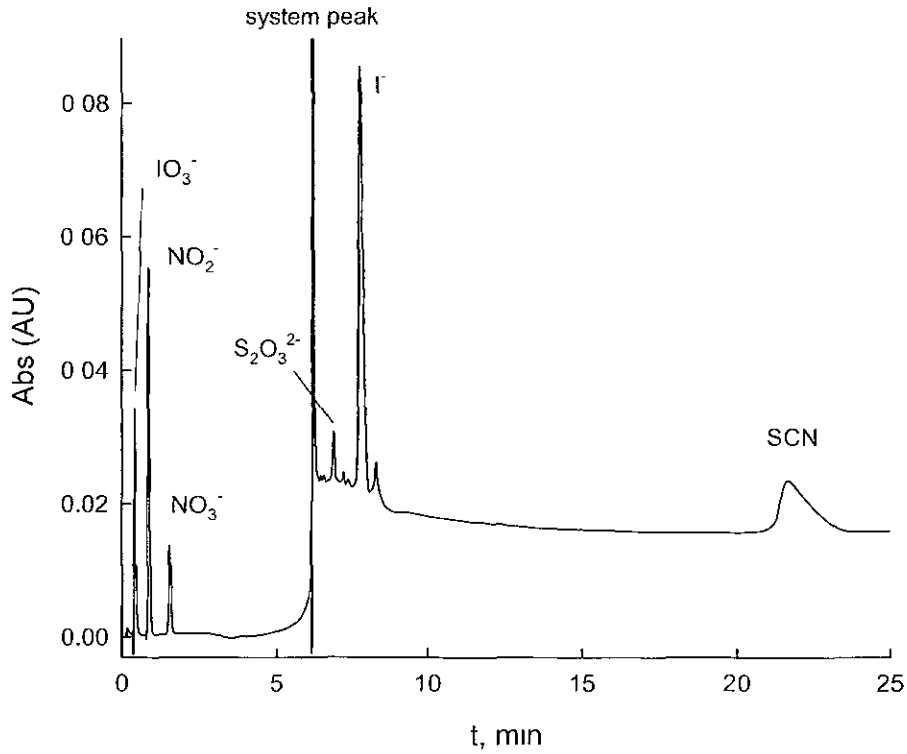


Figure 4-3-5. Chromatogram of standard mixture of six anions. iodate, nitrite, bromide, nitrate, iodide and thiocyanate. Column Gemini C_{18} coated with DDMAU, 50 x 4.6 mm I.D.; Eluents: (A) 10 mM phosphate buffer, pH 2.3, (B) 10 mM phosphate buffer, pH 6 3, F = 1 0 mL/min.
Linear pH gradient: 0.0 - 2 0 min – 100 % A, 2.0 - 5.0 min – 100 % A to 100 % B; 5 0 - 25.0 min - 100 % B

In order to reduce the system peak on the chromatogram, it was decided to increase the ionic strength of the eluent. Therefore, 10 m*M* of KCl was added to each eluent. In Fig. 4-3-6 it can be seen that the system peak was much smaller compared to the one shown in the chromatogram in Fig. 4-3-5, although a change in the baseline could still be seen on the chromatogram. As KCl does not absorb at the selected wavelength, it was suggested that this effect occurs due to the presence of impurities in the initial reagents. As shown before, an increase in the ionic strength of the eluent results in a decrease of the retention time of analyte anions. So for this separation, in order to obtain a better resolution of the weakly retained anions, it was decided to apply isocratic conditions for a longer starting period than for previous separations. After the first five anions were separated, the gradient was applied to rapidly elute iodide and thiocyanate.

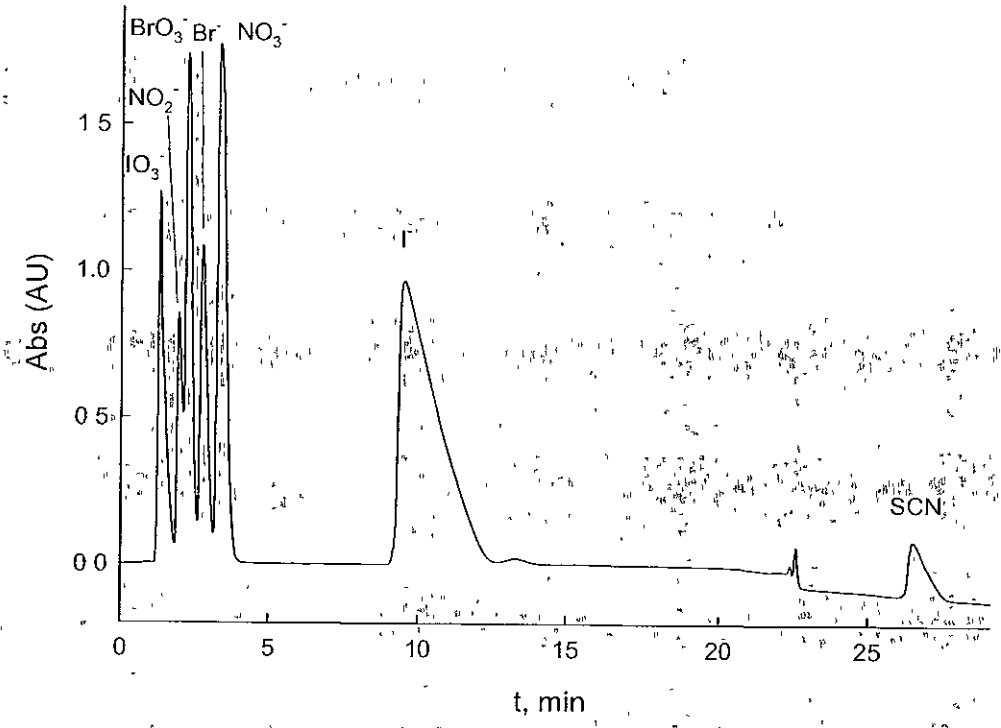


Figure 4-3-6. Chromatogram of standard mixture of six anions: iodate, nitrite, bromate, bromide, nitrate, iodide and thiocyanate. Column Gemini C_{18} coated with DDMAU, 50 x 4.6 mm I.D.; Eluents: (A) 10 mM phosphate buffer, 10 mM KCl, pH 2.3, (B) 10 mM phosphate buffer, 10 mM KCl pH 6.3, F = 1.0 mL/min

Linear pH gradient. 0.0 - 5.0 min – 100 % A, 5.0 - 20.0 min – 100 % A to 100 % B; 20.0 - 30.0 min – 100 % B

4.4. Characterisation of the capacity of the DDMAU – coated column

4.4.1. The monitoring of column eluate pH

To investigate both the pH dependent ion-exchange capacity behaviour of the DDMAU – modified particle packed RP column, and the origins of the system peak observed during application of pH gradients, the rate of change of the column eluate pH was monitored during the application of a pH gradient. This was accomplished through the use of a flow-through pH detector cell, which is shown in Fig. 4-4-1. It was connected to the column outlet after the detector and before eluate was vented to waste.

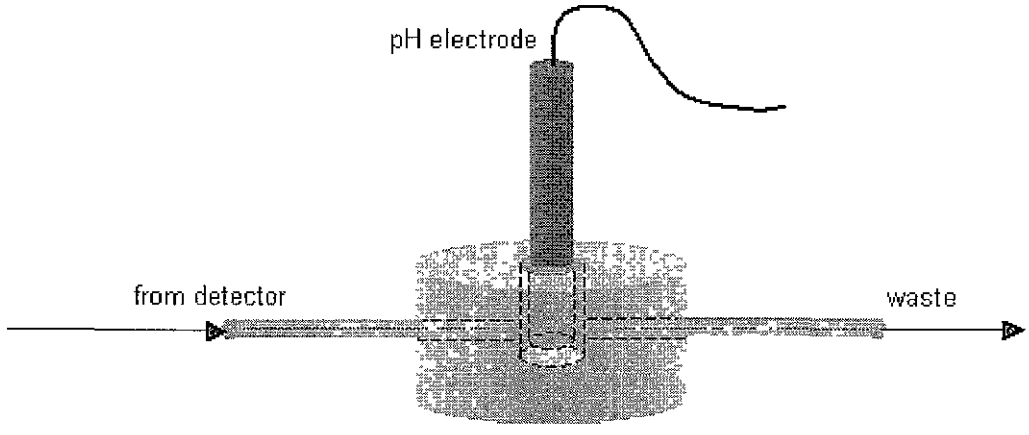


Figure 4-4-1. A flow through pH detector cell

Using ten different pre-programmed gradient profiles from convex to concave, the pH of the column eluate in each case was monitored over the runtime of the chromatogram utilising a constant flow of 1.0 mL/min. The resultant plots of pH versus time for the linear, convex and concave gradient profiles for DDMAU-coated particle packed C_{18} column (50 x 4.6mm I.D.) can be seen in Figure 4-4-2.

The results obtained showed that the DDMAU modified column exhibits a considerable buffering capacity as can be seen from the shape of the pH response. Initially, the change in eluate pH was small, with pH increasing slowly by approximately 0.2 units, before the rapid change in pH up to the end value of pH ~ 6.3 was reached. The reason for this was the column maintained the starting pH conditions until the buffering capacity of the column (due to the presence of weak acid groups on the surface of the stationary phase) was consumed. This was then followed by a more rapid increase in eluate pH, the timing of which corresponds exactly to the observed system peak on the earlier chromatogram (Fig. 4-3-5).

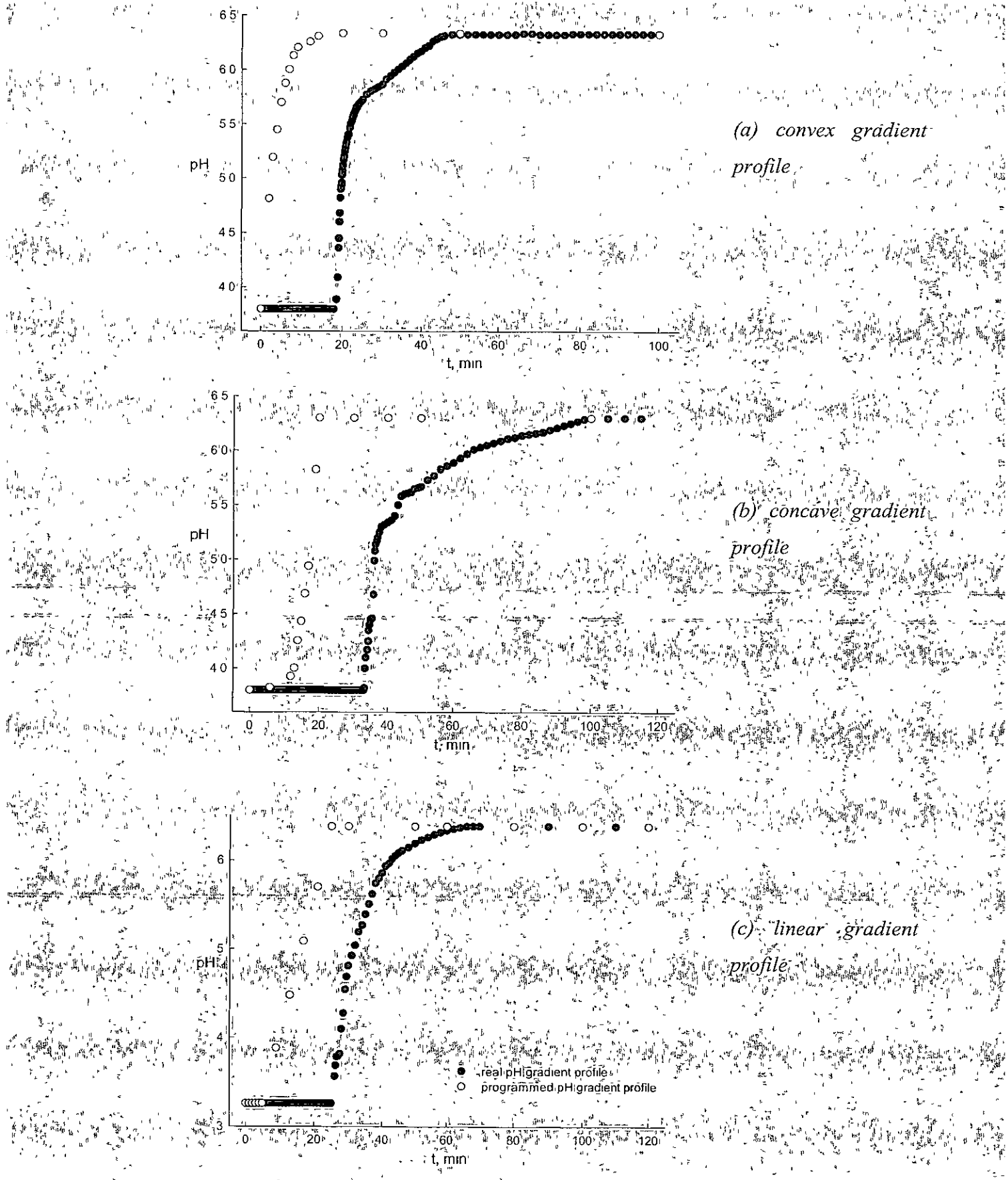


Figure 4-4-2 The comparison of applied and recorded pH gradient profiles. Eluents 10 mM phosphate buffers pH 3.3 and 6.3

In order to obtain a chromatogram without a system peak and with a smoother pH gradient profile, it was decided to raise the ionic strength of the starting and finishing eluents. The starting solution was 10 m*M* phosphate buffer, pH 3.6 and finishing solution was 10 m*M* phosphate buffer pH 6.4. The ionic strength was increased by 0.01 mol/L with KCl for both eluents so the total ionic strength for both solutions was 0.07 mol/L. The resultant plot of pH versus time for the applied linear gradient profile on the DDMAU-coated particle packed column (50 x 4.6 mm I.D.) can be seen in Figure 4-4-3. Unfortunately, the increase in ionic strength resulted in deterioration of the pH gradient profile where the system peak occurred (the same effect was observed for concave and convex pH gradient profiles). The system peak was also still present on all chromatograms.

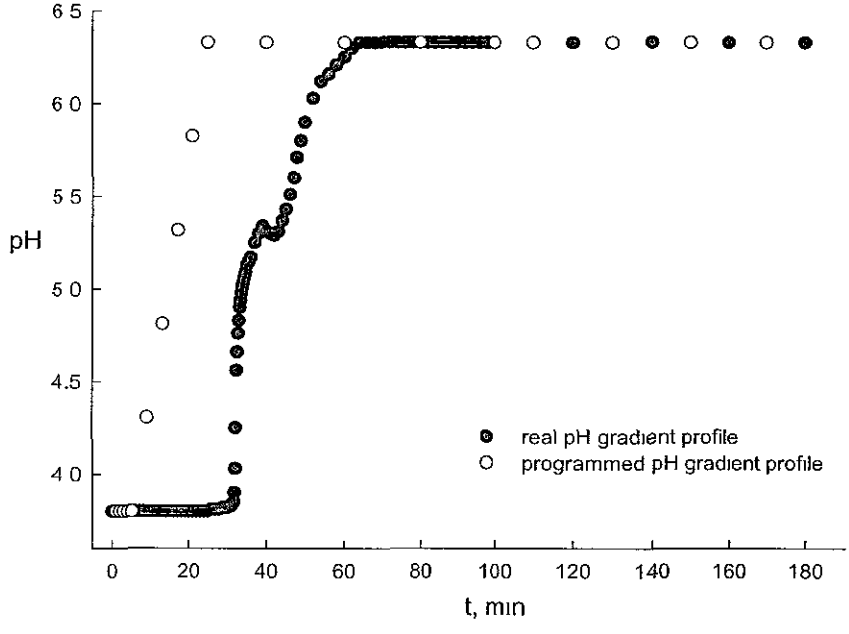


Figure 4-4-3. The comparison of applied and recorded pH gradient profiles. Eluents: 10 mM phosphate buffers, pH 3.6 and 6.4, 10 mM KCl

The next step was the utilisation of a citrate buffer to create a smoother pH gradient profile. For this, 10 m*M* citrate buffer, pH 3.17 was used as starting solution and 10 m*M* citrate buffer, pH 6.24 was used as finishing solution. The linear gradient was applied and the resultant plot of pH versus time for the linear gradient profile can be seen in Figure 4-4-4. Though the profile of pH gradient was more linear when using citrate buffer, on the resultant chromatograms a sharp rise at the time corresponding to the inflection point of the curved area on the pH gradient profile was observed. The reason for that might be an unsuitable eluent, as citrate absorbs at the selected wavelength of 214 nm, or the presence of contaminants in the initial reagents used for buffer preparation.

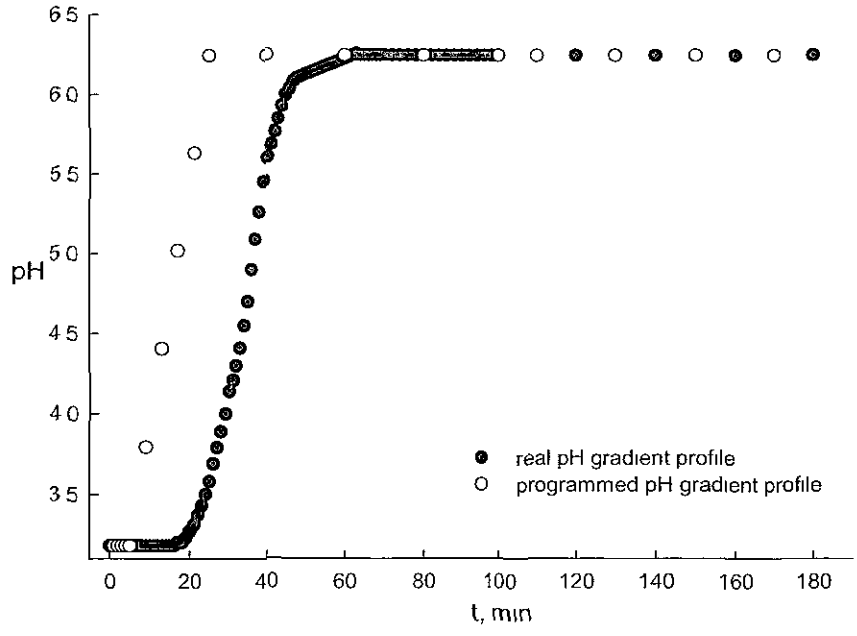


Figure 4-4-4 The comparison of linear programmed and real pH gradient profiles Eluents: 10 mM citrate buffers, pH 3 17 and 6.24

4.4 2 Determination of column capacity

In order to estimate column ion-exchange capacity for particle packed and monolithic RP columns coated with DDMAU, a modified version of the experiment developed by Hendricks *et al.* [14] was undertaken. Initially the column was coated with the surfactant and then equilibrated with 5 m*M* phosphate buffer pH 3.0 to ensure that all carboxylic groups of adsorbed surfactant molecules were protonated. The column outlet pH was recorded to ensure column equilibration at pH 3.0 was complete. Then a step pH gradient was applied by washing the column with 5 m*M* phosphate buffer solution at pH 6.0. The pH gradient was carried out twice: first with unmodified column in order to exclude the influence of equipment dead volume and then again with DDMAU coated one. The resulting capacity was estimated by subtraction of the first gradient from the second. To calculate the column capacity the system dwell volume should be known. The dwell time, t_{dwell}, is the time from the point of inception of the eluent step gradient to the tangent that extends through the inflection point of the curved area of the pH plot and can be calculated from eq. 2-4-1.

4.4.2.1 Particle packed RP column coated with DDMAU

The initial experiment was performed with an unmodified particle packed RP 50 x 4.6 mm I.D. column. Using eq. 2-4-1 the calculated value for V_{dwell} was found to be 1.2 mL (at 1.0 mL/min flow rate). Repeating this experiment with the DDMAU modified column allowed calculation of the buffering capacity of the column and thus its effective anion exchange capacity. A comparison of the column eluate pH using a 1

min long linear pH gradient on an unmodified column with the coated column can be seen in Fig. 4-4-5. With a known concentration of phosphate eluent of 5 m*M* and a flow rate of 1.0 mL/min, the effective ion-exchange capacity of the modified column was determined to be 54 μmol. This value is approximately the same (48 μmol) as calculated during the column coating procedure (at the breakthrough point).

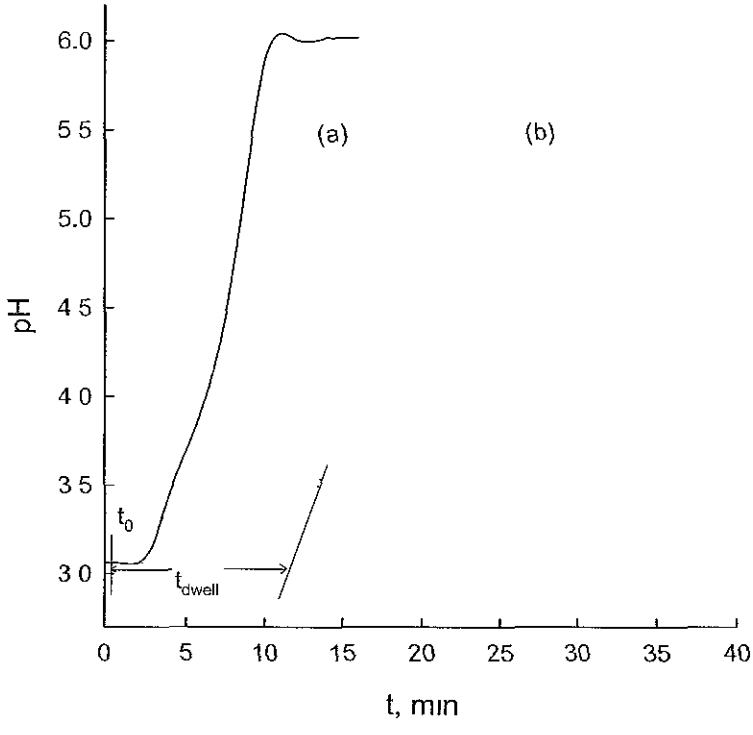


Figure 4-4-5. A comparison of the column eluate pH using a linear pH gradient for an unmodified column with a coated one pH gradient profiles for Gemini C_{18} (a) uncoated column, (b) DDMAU modified column. Eluents: A – 5 mM phosphate buffer, pH 3.1; B – 5 mM phosphate buffer, pH 6.1. Linear pH gradient: 0.0 – 1.0 min 100 % A to 100 % B

4.4.2.2. Monolithic RP column coated with DDMAU

The same experiment was performed for the monolithic RP 100 x 4.6 mm I.D. monolithic column. First the experiment was run with an unmodified column and the V_{dwell}, calculated according to eq. 2-4-1, was found to be 3.3 mL (at 1.0 mL/min flow rate). After this the experiment was again repeated with a modified column. A comparison of the column eluate pH using a 1 min long linear pH gradient of unmodified column with a coated column can be seen in Fig 4-4-6. With a known concentration of phosphate buffer of 10 m*M* and the flow rate of 1.0 mL/min, the effective column capacity of the modified column was determined to be 99 μmol. This value is slightly below the experimentally determined value of 110 μmol of DDMAU, which was calculated for the column coating (at the breakthrough point). This was anticipated as not all of the DDMAU molecules attached to the surface would be available to take part in the anion exchange process.

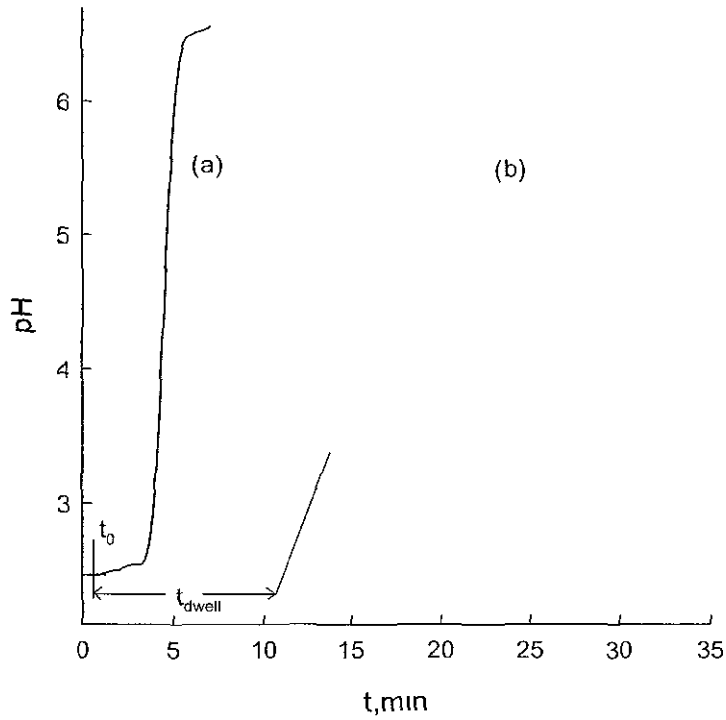


Figure 4-4-6. A comparison of the column eluate pH using a linear pH gradient for an unmodified column with a coated one. pH gradient profiles for Chromolith RP (a) uncoated column, (b) DDMAU modified column. Eluents: A – 10 mM phosphate buffer, pH 2 6; B – 10 mM phosphate buffer, pH 6 5. Linear pH gradient: 0.0 – 1.0 min 100 % A to 100 % B

4.5. Separation of anions under flow gradient mode using a DDMAU coated micro-column

In HPLC and IC in many application areas, there has recently been a move away from traditional length analytical columns to shorter columns, packed with highly efficient stationary phases (3-5 μm), with the aim of achieving shorter analysis times, lower sample and solvent consumption, faster re-equilibration and high mass sensitivity [15-17]. As the efficiency of a column is inversely proportional to the particle size, longer, more traditional columns with larger particles may be substituted for shorter columns with smaller particles without sacrificing too much in terms of resolution, performance and reliability [18]. Moreover, the application of short columns can result in simplification and miniaturisation of chromatographic instrumentation. For example, recently short monolithic columns, from 10 to 25 mm length, have been used in conjunction with simple peristaltic pumping systems for low-pressure ion chromatography [19,20]. Several fundamental and applied studies into the use of short particle packed RP-HPLC columns (from 3 to 50 mm in length) have emerged in recent years, predominantly based upon 3-5 μm ODS stationary phases [21,22], which highlight their potential for rapid separations of relatively simple sample mixtures.

In the area of ion-exchange chromatography there has been less interest in exploring the type of separations possible using such ultra-short columns. This may be

due in part to the fact that the majority of commercial ion-exchange phases are polymer based, due to increased pH stability, and tend to exhibit relatively poorer peak efficiencies when compared to those obtainable in RP-HPLC using the above silica-based materials.

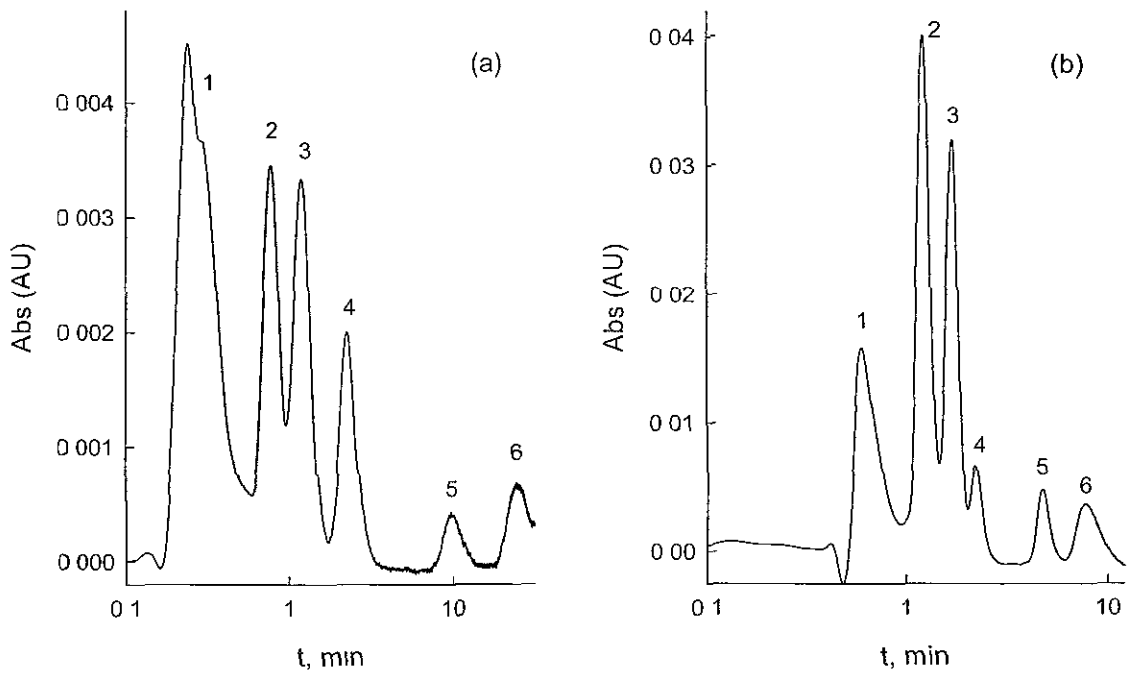
The use of ultra-short analytical columns permits the utilisation of elevated flow rates (up to 5 mL/min) to also counter excessive retention times and allows the column to be used under flow gradient conditions, similar to those shown to be effective when using short monolithic columns [11].

4.5.1. Application of a flow gradient profile

As mentioned above, an advantage in using short columns is reduced analysis times. However, the relatively low column backpressures with such short columns also allow the application of elevated flow rates to reduce run times still further. Such elevated flow rates can be utilised at a constant set rate, so-called isofluentic mode, or within an applied flow programme. However, the approach of flow programming has had very limited application in HPLC separations due to sharp increases in backpressure associated with steep flow gradients. Consequently, the choice of flow rates for such a profile is confined to a relatively narrow range. However, the application of flow gradients over a wider flow range, as is possible with shorter columns, can produce chromatographic results similar to those resulting from eluent concentration gradients, without the need for a complex gradient pumping system.

A DDMAU modified micro-column (SecurityGuard Gemini C_{18}, 4.0 x 3.0 mm I.D.) was used to compare a chromatogram obtained using a flow gradient with one that was achieved under isofluentic conditions. Flow rates of between 0.3 and 5.0 mL/min were investigated using the column, with the 5.0 mL/min flow rate resulting in a moderate backpressure of less than 77 atm. Under isofluentic conditions of 1.0 mL/min, a mixture of six anions could be separated in approximately 35 min. However, peak widths (w) for both thiocyanate and trichloroacetate were excessively broad at $\sim w = 5$ min and $\sim w = 12$ min respectively. This chromatogram is shown at Fig. 4-5-1a. Increasing the column flow rate to a constant flow of 2, 3 or even 5 mL/min would result in the obvious reduction in total run times but would also produce a reduction in peak efficiency for all anions, causing coelution of the least retained most closely eluting anions. For the stronger retained and better resolved anions this decrease in efficiency seen at elevated flow rates did not result in such coeluting peaks.

Therefore, the same mixture was run with a flow gradient from 0.3 to 5.0 mL/min over the first 3 min and then maintained at 5.0 mL/min for a further 9 min. The resultant chromatogram under these conditions is shown in Fig. 4-5-2b. Under these conditions, resolution of the early eluting anions is maintained, although still not completely baseline resolved, and the overall run time was reduced to a little over 10 min. This represents an approximate 70 % reduction in run time without significant loss of overall resolution. Also peak widths for thiocyanate and trichloroacetate were now reduced to w = 2 min and $\sim w$ = 3.5 min, respectively. In addition to this, there was no disturbance observed in the baseline related to the rather steep flow gradient.



(a). Isofluentic conditions F = 1.0 mL/min

(b) Flow gradient conditions.
0 0 – 3 0 min – 0 3 mL/min to 5 0 mL/min
3.0 – 12.0 min – 5.0 mL/min

Figure 4-5-1. Separation of a standard of six anions. Elution order: 1 – nitrite, 2 – nitrate, 3 – benzoate, 4 – iodide, 5 – thiocyanate, 6 – trichloroacetate. Column: SecurityGuard Gemini C_{18}, 4.0 x 3.0 mm I.D , coated with DDMAU Eluent in (a) and (b) 10 mM phosphate buffer, pH 2.2

4.5.2 Effect of flow gradient and sample ionic strength on peak efficiency and retention time stability

A flow gradient separation can be considered in a similar way to a simple isocratic separation, since a constant composition of eluent is used. Theoretically, under conditions of sufficiently fast mass transfer kinetics, this should mean a constant

distribution coefficient exists for each analyte in such a chromatographic system, and thus allow some observations on peak efficiencies to be made, particularly to directly compare the efficiencies shown by the flow gradient and the isofluentic separations. To calculate such efficiency data, retention time was replaced by retention volume and peak width was calculated as the volume of eluent passed through the column during the period of time equal to the peak width [11,22]. With simple standard solutions it was observed that for each anion the use of the flow gradient approach resulted in a minor improvement in efficiency in comparison with isofluentic elution. With the isofluentic approach, 50 mg/L standards resulted in efficiency values of $N = 58$, $N = 67$ and $N = 56$ for nitrate, iodide and thiocyanate, respectively (~14 000 – 17 000 N/m). These compare very favourably with values of $N = 13$, $N = 12.5$ and $N = 12.3$, reported previously for a 3-mm long polymer porous monolithic anion-exchanger [23]. At the same time, work by Yan *et al.* [24] has suggested that the coating procedures used when modifying RP materials using ionic surfactants, and also the use of mixed ionic and nonionic surfactant coatings, can result in significant improvements in efficiency. This approach could also be applicable for the current system with DDMAU-coated micro-column to improve efficiencies still further.

However, it was observed that for standards prepared in more complex matrices, such as increasing NaCl content, the observed differences in peak efficiency for certain anions were more pronounced and less predictable. Therefore, 20 mg/L standards were spiked with 0 to 500 mM concentrations of chloride (where $n = 8$). For nitrite, nitrate and thiocyanate some advantage in terms of peak efficiency when using the flow gradient approach with samples of higher ionic strength (NaCl) appeared. For iodide there was no advantage at higher ionic strengths, and for trichloroacetate the presence of NaCl in the sample resulted in poorer peak efficiency when a flow gradient was applied. The different behaviour during the flow gradient shown by trichloroacetate is obviously due to a different retention mechanism between trichloroacetate and the coated RP column, which would include more significant hydrophobic interactions than for inorganic anions. The comparison of the calculated efficiencies (N) with and without the applied flow gradient is shown in Figure 4-5-2. Peak efficiencies were determined under isofluentic conditions over an analyte (nitrite, iodide and thiocyanate) concentration range of 1 – 1000 mg/L ($n = 10$, standards were prepared in water only). As it was expected, there was a small decrease in peak efficiency with increasing analyte concentration. With regard to retention time stability over this concentration range, nitrate was least affected (1.1 % RSD), followed by iodide (3.3 % RSD), followed by

thiocyanate (6.6 % RSD). Additional standards of 2, 5, 7.5 and 10 g/L of thiocyanate were prepared and it was found that retention time decreased from 8.5 to 3.5 min over to complete 50 – 10 000 mg/L range, indicating that at such high concentrations column capacity was being exceeded. Table 4-5-1 lists the retention factors and efficiency data for the above study.

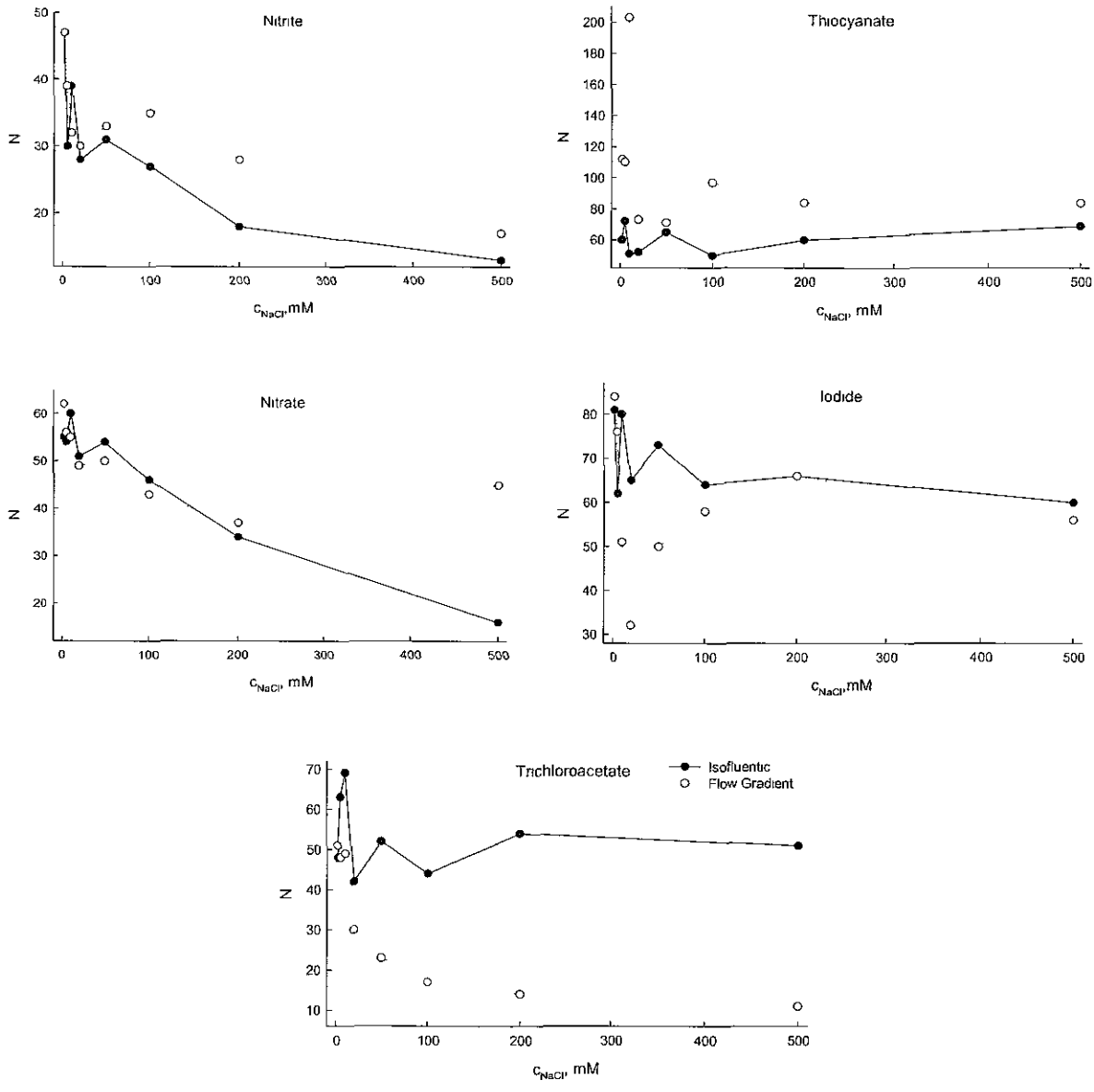


Figure 4-5-2. Comparison of peak efficiencies for isofluentic and flow gradient separations of nitrite, nitrate, iodide, thiocyanate and trichloroacetate under increasing sample NaCl content

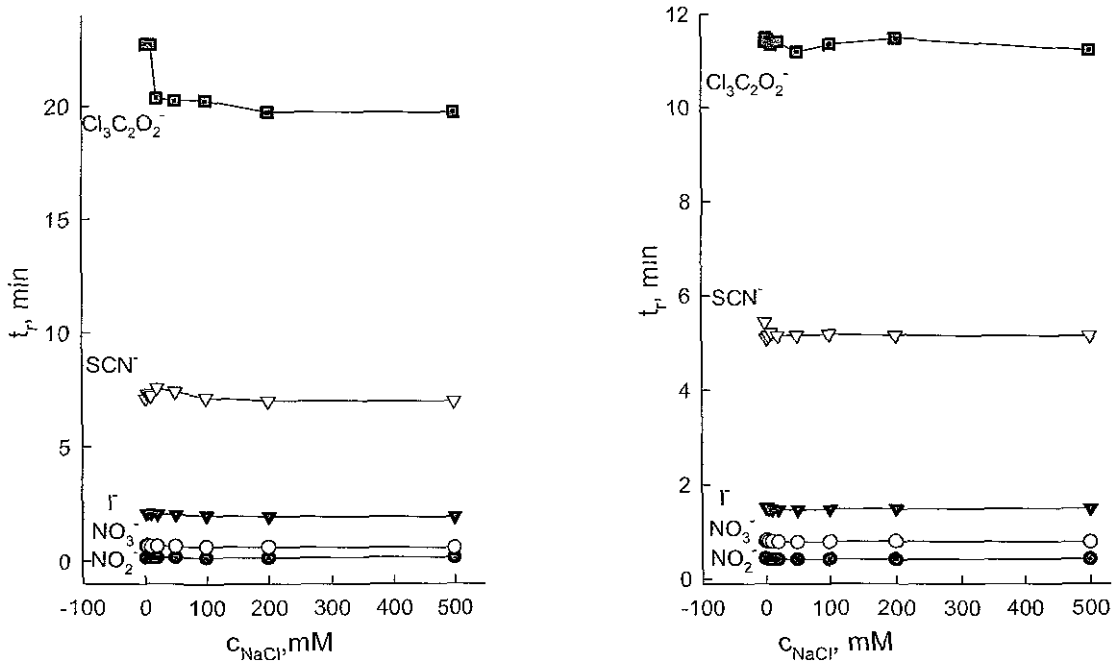
Table 4-5-1 Influence of sample concentration on retention factor and peak efficiency (for the column length 4 mm)

c (mg/L)	*k*, NO_3^-	*k*, I^-	*N*, NO_3^-	*N*, I^-	*c* (mg/L)	*k*, SCN^-	*N*, SCN^-
1	1.88	-	55	-	50	55.37	56
2	1.83	11.19	56	74	200	53.13	52
5	1.89	11.36	56	70	500	50.62	58
10	1.92	11.2	57	68	1000	47.27	50
20	1.96	11.02	57	67	2000	41.22	35
50	2.01	10.9	58	67	5000	32 6	21
100	1.99	10.76	57	67	7500	27.27	16
200	1.99	10.55	56	67	10 000	22.53	12
500	1.99	10.3	55	66			
1000	1.88	9 98	52	63			

The retention time stability with increasing sample NaCl content was investigated. Increasing ionic strength using NaCl caused little or no change in retention times under both isofluentic and flow gradient conditions for most anions, except trichloroacetate, where retention did initially decrease before stabilising under isofluentic conditions. For the remaining anions retention time stability was remarkably good given the dimensions of the micro-column and the high NaCl content within the standard, up to 500 m*M* (17 725 mg/L Cl^-). These results reflect the low affinity of the DDMAU phase to Cl^- ions making the micro-column potentially suited to the rapid analysis of saline samples. Figure 4-5-3 illustrates retention time stability with increasing sample ionic strength under both flow modes, and again shows the reduction of overall run time obtained using the flow gradient approach.

4.5.3. Column capacity

Overall column capacity was determined using an applied eluent pH gradient over the range pH 3.06 and 6 02 and monitoring the pH of the column eluate. From the resultant pH data the total buffering capacity of the column can be used to calculate adsorbed DDMAU and effective column ion-exchange capacity. The eluent pH gradient was applied with coated and uncoated columns to eliminate any delay due to system dwell time from the capacity calculations, according to the method of Hendricks *et al* [14] (as described in Section 2.4.5). This method produced an effective column capacity of 16 μmol.



(a) Isofluentic

(b) Flow gradient

0 0 – 3 0 mın - from 0.3 to 5 0 mL/min

3.0 – 12 0 min – 5.0 mL/mın

Figure 4-5-3. Dependence of retention time on the ıonic strength

4.5 4. Analytical performance characteristics

Linearity for nitrate, ıodide and thiocyanate was investigated with the respect to peak heıght (mAU). Calibration standards were prepared ın the range 1 – 1000 mg/mL and injected in triplicate. It was found that the micro-column exhıbited excellent linearity over thıs concentration range with correlation coefficients above $R^2 > 0.996$ across the complete range of calibration coefficıents studied. The results are presented ın the Table 4-5-2.

Table 4-5-2. Analytical performance characteristics for selected anions

Anion	R^2	Slope	Intercept	LOD mg/L
NO_3^-	0.998	0.427	2.308	0.1±0.004
I^-	0.999	0.044	0.271	0.2±0.014
SCN^-	0.996	0 022	-3.434	10.0±1.871

Detection lımits were calculated for each of the three anions by serially diluting standards of the three anions to a point where signal to noise ratio became close to 3:1. The injection volume for all analytes solutions was 2 µL.

4.5.5. Analysis of iodised salt

The DDMAU modified micro-column was applied to the analysis of a commercially available iodised salt (Saxa, RHM, Middlewich, Cheshire, UK). Previous attempts at the determination of iodide in NaCl brine solutions have utilised 250-mm-long columns with relatively high anion exchange capacity, in conjunction with complex pulsed amperometric detection. This approach led to the trace iodide peak eluting on the tail of a large chloride matrix peak, with a run time of between 6 and 10 min [25] The method was also not suitable for the simultaneous detection of iodide and iodate in such a complex matrix.

Here, an exact weight of the sample was dissolved into ultrapure water to give a final concentration of 20 g/L. The salt solution was filtered and injected onto the micro-column under isofluentic conditions (1 mL/min) with a 10 m*M* phosphate buffer, pH 2.22. In the sample, three anions were found present, namely, chloride, iodate and iodide, with the large excess of chloride seen as a sharp peak (but relatively small due to low chloride absorbance at 210 nm) eluting close to the column void time of ~ 0.2 min. Given the relatively high concentrations of these anions in the sample, there was no significant interference from other UV-absorbing anions, such as possible trace levels of bromide and nitrate. The resultant chromatogram is shown at Fig. 4-5-4. The extremely high NaCl matrix resulted in a significant system peak eluting at 0.6 min, although this did not interfere with the analyte anion peaks. This system peak was due to the use of phosphate eluent and was only seen with high salinity samples. The system peak could be removed completely with the use of chloride based eluents, although the selectivity of the column was such that buffering eluents were required and chloride was also a very weak eluent anion. The small but significant peak for iodate is to be expected from iodised table salt through oxidation and may have been purposely added to limit further oxidation of the iodide during storage. As can be seen from Fig 4-5-4, iodide was completely resolved from the high-level matrix chloride peak and could be determined with little or no interference (the relatively low absorbance for chloride at 210 nm being a distinct advantage for this specific application).

The efficiency of this iodide peak was calculated to be 72 theoretical plates (18 000 *N*/m). The peak for iodate was only partially resolved from chloride peak, although was clearly identified through standard addition. Both iodide and iodate were quantified by standard addition calibration, firstly by carrying out $n = 6$ replicate injections of the neat salt solution. Fortifications were made by spiking eleven duplicate aliquots of this solution with 0.3 – 5.0 μ*M* iodide and 0.1 - 2.0 μ*M* iodate, respectively. An overlay of

spiked solutions is represented in Fig 4-5-5 and there were no significant decreases in retention

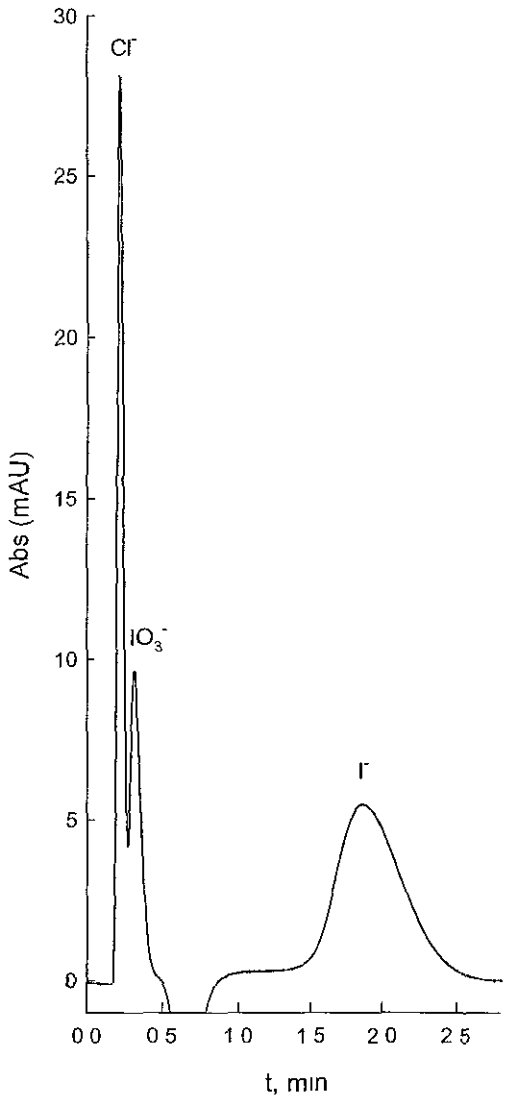


Figure 4-5-4. Separation of iodide and iodate from high levels matrix chloride in a 20 g/L solution of commercial iodised table salt.

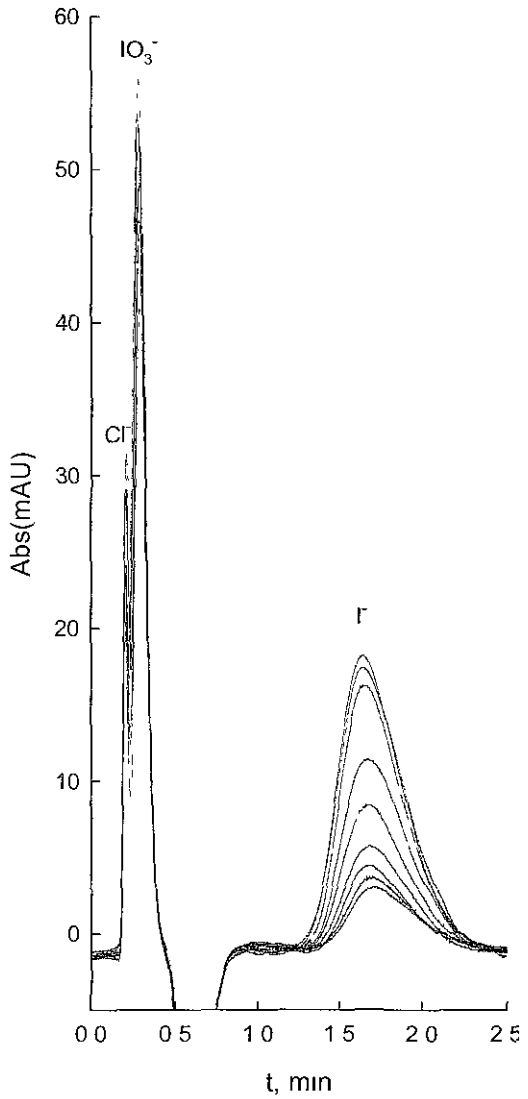


Figure 4-5-5. Overlaid chromatograms of a 20 g/L iodised salt sample spiked with between 0,3 and 5.0 μM iodide and 0.1 and 2.0 μM iodate.

Column: Gemini C_{18} coated with DDMAU, 4.0 x 3 0 mm I.D. Eluent 10 mM phosphate buffer pH 2.2, F=1.0 mL/min

observed with increasing iodide or iodate concentration. Taking into account any dilution factors, extrapolation of the standard addition curve yielded 2.80 ± 0.04 μ*M* iodide ($R^2 = 0.974$) and 0.60 ± 0.01 μ*M* iodate ($R^2 = 0.991$) calculated for a 100 g/L salt solution, which in turn corresponded to 3.55 ± 0.05 μg I^-/g and 1.05 ± 0.02 μg IO_3^-/g of neat salt sample.

4.6. Conclusions

The effect of eluent concentration and pH on separation of anions on particle packed and monolithic RP columns coated with either DDMAU or DDMAB was studied. The separation conditions for each column were optimised using product resolution criterion and resultant response surface. An attempt to apply a pH gradient to the separation was made and investigation of column capacities has been carried out. The investigation of a short DDMAU micro-column for ion chromatographic separations has been carried out. The stable zwitterionic DDMAU coating resulted in a relatively high capacity column, which can be used as an anion exchanger for the rapid separation of a limited number of anions. The use of a flow gradient approach to reduce excessive run times could be applied to the micro-column due to the relatively low backpressures generated over a column of such short dimensions. Micro-column coated with DDMAU exhibited stable retention times and linearity over an impressive range of analyte and matrix anion concentrations, and a low affinity to chloride allowed the column to be applied directly to saline samples.

4.7. Reference list

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CHAPTER 5. SIMULTANEOUS SEPARATION OF INORGANIC ANIONS AND CATIONS ON ZWITTERIONIC STATIONARY PHASES

Since its introduction in 1975 by Small [1], ion chromatography (IC) has emerged as a powerful method for the analysis of anions and cations in a variety of environmental samples, especially in natural and industrial water [2]. Usually the cations and anions have to be determined separately with different chromatographic columns, eluents and even instruments. However, there are a few approaches that can be used to achieve simultaneous separation and determination of negatively and positively charged ions from a single injected sample. These approaches are of great interest, as the simultaneous determination of inorganic anions and cations can result in a substantial time and economic savings.

According to the accepted classification [3,4] all methods for the simultaneous separation and determination of inorganic cations and anions can be divided into multi-column and single-column systems. Multi-columns systems are relatively complex and include:

1. Parallel columns determination of cations and anions [5].
2. In-series anion- and cation-exchange columns with the use of single or dual detection systems [6-8].
3. Systems incorporating column switching capabilities.

Single column systems for the simultaneous separation of anions and cations have been based upon the ability of zwitterionic ion-exchangers [9-11], agglomerated ion-exchangers [10] or mixed bed packings comprised of anion- and cation-exchangers [9], cation-exchange/ion-exclusion mechanisms in carboxylic acid cation-exchangers to retain oppositely charged ions [11], or complexation to convert cations to negatively charged complexes to be separated by purely anion-exchange chromatography. The latter approach has the advantage of simplicity and of using standard chromatographic columns for anion chromatography.

The simultaneous separation of anions and negatively charged complexes of metals is based on conversion of the effective charge of alkaline-earth and transition metal ions from positive to negative by complexation with various aminocarboxylic ligands. Matsushita and Yamamoto [12,13] in their pioneering work explored the ability of alkaline-earth and transition metal cations to form negatively charged complexes with ethylenediaminetetraacetic acid (EDTA). Lately, other complexones including 1,2

– diaminocyclohexanetetraacetic acid (DCTA) [14-16], ethyleneglycol-bis2(2-aminoethylether) – *N,N,N',N'* – tetraacetic acid (EGTA) [14,15] and diethylenetriaminopentaacetic acid (DTPA) [14] were also investigated as additives to ion chromatographic eluents. The separation of up to ten anions, alkaline-earth and transition metal cations including calcium, cadmium (II), manganese (II), cobalt (II) and zinc (II), utilising UV detection, was achieved. With additives of DCTA to the eluent the separation of common anions and transition metals of different valency including copper (II), mercury (II), cobalt (II), and molybdenum (VI) on a silica-based anion-exchange column has been obtained [16]. It was shown that the eluent strength of the complexones investigated increased as follows: EGTA < DCTA < EDTA < DTPA. However, EGTA and DTPA were not suitable for the separation of cations due to peak broadening as a result of slow chelating kinetics. Despite rather broad peaks, EDTA is still the most common eluent for the simultaneous determination of anions and anionic complexes of metals [17-20].

The ion-exchange selectivity for the separation of metal complexes with polyaminocarboxylic ligands depends on competing complex formation and acid-base equilibrium [15,21-23]. As pH of the mobile phase increases, the dissociation of the polyaminocarboxylic ligand occurs and leads to the formation of more strongly retained ML^{2-} complexes. Further increases in the eluent pH contributes to the competing formation of metal hydroxo-complexes, while the elution strength of the eluent increases due to dissociation of its weak acid components. Thus, whereas the decrease in the retention for inorganic anions is relatively straight forward, a bell shape dependence of retention on eluent pH is typical for negatively charged metal complexes.

A disadvantage of polyaminocarboxylic acids is the relatively poor kinetics of complexation with metals, such that pre-column formation of complexes is often required [12-15,17,20] to achieve reasonable separation efficiency and resolution. However, there are a few reports of the application of other ligands for anion chromatography of anions and negatively charged complexes. Ohta and Tanaka [24,25] developed the simultaneous separation of common inorganic anions and divalent cations using 1,2,4,5–benzenetetracarboxylic acid or pyromellitic acid [24,25] as the eluent. Chen and Adams used dipicolinic and trimesic acids as eluents [26] for the separation of inorganic anions, magnesium and calcium with indirect UV or conductivity detection. Haddad *et al.* [27] presented the simultaneous determination of complexes of lead (II), copper (II) and nitrate on a silica based anion-exchange column with citrate – oxalate eluent and indirect potentiometric detection.

According to the literature the ideal complexing agent in the eluent for the simultaneous separation of anions and negatively charged metal complexes should fit the following requirements:

1. They should form stable negatively charged complexes with various alkaline-earth and transition metal ions. The stability of the complexes should be sufficient to suppress secondary undesirable chemical equilibria in the chromatographic system like hydrolysis of metals;
2. Ideally, the kinetics of complexation should be fast enough for on-column formation of negatively charged complexes to avoid their pre-column synthesis;
3. Complexes should be soluble.

Citric acid is well suited to these demands. However, interestingly citric acid eluents have not been used for the simultaneous separation of anions and anionic complexes of alkaline-earth and transition metal cations [7,28-30].

The main focus of this Chapter of work was an investigation into the retention and separation selectivity of anions and negatively charged citrate complexes on RP stationary phases, dynamically coated with zwitterionic surfactant *N*-(dodecyl-*N*,*N*-dimethylammonio)undecanoate. DDMAU coated RP sorbents exhibit mainly anion-exchange properties in acidic eluents, but the zwitterionic nature of the coating cannot be completely excluded and may impact in ion-exchange selectivity.

5.1. Effect of the eluent pH on the retention of metal cations

RP materials dynamically modified with carboxybetaine surfactants have been shown as efficient anion-exchangers in separation of inorganic anions [31-33]. There are three possible ways in which pH of the organic acid (citric, oxalic or acetic) based eluent may affect on retention and separation selectivity of ions. Firstly, the increase in pH causes the dissociation of the carboxylic groups from the adsorbed DDMAU molecules (pK_a = 5.04), which affects the ion-exchange capacity of this type of ion-exchanger and, hence, the retention of inorganic anions. Secondly, the dissociation of carboxylic groups in citric (pK_{a1} 3.13; pK_{a2} 4.76; pK_{a3} 7.40), oxalic (pK_{a1} 1.20; pK_{a2} 4.20) or acetic (pK_a = 4.76) acids within the eluent increases the charge of the eluting anion and, hence, the elution power of these eluents. Finally, the conditional stability constant of the metal complexes (Table 5-1-1) formed with these acids and the charges of the complex ions are also pH dependant. Therefore, the resulting effect of eluent pH on the retention of complex anions cannot be simply predicted, and so the effect of eluent pH on the retention of metal ions was investigated for the particle packed Gemini

C_{18} (Phenomenex) column coated with DDMAU. 1 m*M* citrate, oxalate or acetate buffers with pH adjusted from 4.0 to 7.0, were used as test eluents.

Table 5-1-1. Stability constants of citrate and oxalate metal complexes [34]

Log stability constant of ML complexes (alkali and alkaline-earth metals)									
Acid	Li	Na	K	NH_4	Cs	Mg	Ca	Sr	Ba
Citric	0.83	0.75	0.60	-	0.32	3.43	3.48	3.02	2.79
Oxalic	-	0.50	0.33	0.60	-	3.43	3.12	2.54	2.33
Acetic	0.13	-0.27	-0.43	-	-0.33	0.51	0.55	0.47	0.44

Log stability constant of ML complexes (transition metals)							
Acid	Mn(II)	Co(II)	Zn(II)	Fe(III)	Cd(II)	Cu(II)	Ni(II)
Citric	3.76	4.90	4 93	11.19	3.76	3.70	5.18
Oxalic	3.00	3.84	4.00	7.53	3.89	4.85	6.16
Acetic	0.80	0.86	1.07	3.60	1.52	1.79	0.88

It was shown in Chapter 4 that in case of ion-exchangers prepared by dynamic modification of RP materials with carboxybetaine surfactants that the eluent pH has a more pronounced effect on the retention of anions than for ion-exchangers prepared by modification with simple cationic surfactants. This is due to the synergetic effect caused by dissociation of carboxylic groups in adsorbed DDMAU molecules and the organic acids used as the eluent. The increase of the eluent pH simultaneously decreases the ion-exchange capacity and increases the elution power of the eluent. The dissociation of carboxylic groups in the adsorbed DDMAU molecules can be noted from the corresponding pH dependences of the retention of alkali metal cations with an oxalate eluent as shown in Fig. 5-1-1b The observed retention order of alkali metal cations with an oxalate eluent at pH 4.0 corresponds to traditional cation-exchange selectivity, namely:

$$Li^+ < Na^+ < NH_4^+ < K^+ < Cs^+.$$

However, this differs to that observed with a citrate eluent at pH 4.0:

$$Cs^+ \approx Na^+ < K^+ < NH_4^+ < Li^+.$$

The explanation for such a difference in selectivity lies in the higher complexing ability of citrate buffer. Stability constants of negatively charged species of citrate with alkali metal cations (Table 5-1-1) increase from Cs^+ to Li^+, providing a reversal in ion-exchange selectivity due to the mixed mode retention mechanism unique to the

zwitterionic phase. Nevertheless, according to Fig. 5-1-1, all of alkali metal cations were only weakly retained under these conditions, and no separation could be achieved.

A similar difference in ion-exchange selectivity between oxalate and citrate eluents was observed for alkaline-earth metals. Again, the retention order $Mg^{2+} < Ca^{2+} \approx Sr^{2+} < Ba^{2+}$ obtained for the oxalate eluent is typical for carboxylic type cation-exchangers and was the reverse of that seen for the citrate eluent (Fig 5-1-1a). This is in a good agreement with the fact that oxalic acid can form only neutral or slightly positively charged 1:1 complexes/salts with alkaline-earth metals. This means that the alkaline-earth metal cations are retained purely due to cation-exchange interactions with the carboxylic groups from DDMAU The slight increase in retention for alkaline earth metal cations with an increase in eluent pH was observed due to the dissociation of the carboxylic groups in the adsorbed DDMAU molecules.

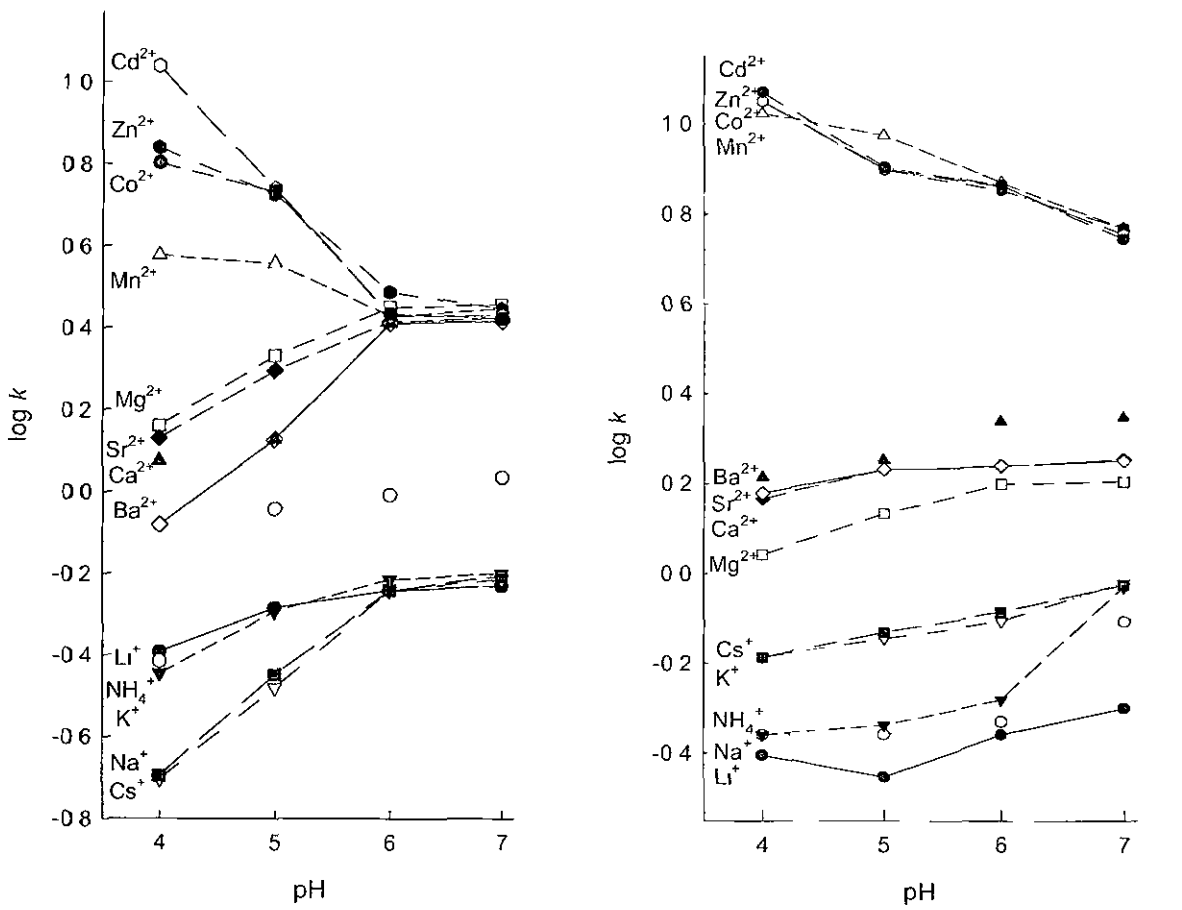

Figure 5-1-1 Dependence of retention factor for selected cations on eluent pH. Column: Gemini C_{18}, coated with DDMAU, 100 x 4 6 mm I.D. Eluent: (a) 1 mM citrate buffer, (b) 1 mM oxalate buffer

The stability constants of alkaline earth metal complexes with citrate and oxalate are of the same order of magnitude (Table 5-1-1). However, tribasic citric acid can form negatively charged 1:1 or 1:2 complexes with these metals, so the retention of alkaline-earth metal cations could be due to anion-exchange interactions. Therefore, the retention of these metals with the citrate eluent at pH 4.0 is proportional to their stability constants, resulting in the elution order $Ba^{2+} < Ca^{2+} \approx Sr^{2+} < Mg^{2+}$. The retention times are increased as the effective negative charge increases with eluent pH.

Significantly stronger retention was observed for transition metals and a decrease in retention with an increase of eluent pH was observed for both types of eluents. As transition metals can form anionic complexes with both citric and oxalic acid, these complexes interact with the DDMAU-coated zwitterionic stationary phases based upon an anion-exchange mechanism, similarly to inorganic anions, for which the retention decreases with an increase in pH. The retention order for transition metals was the same for both eluents at pH 4.0:

$$Mn^{2+} < Co^{2+} \approx Zn^{2+} < Cd^{2+}.$$

However, selectivity was higher for the citrate buffer based eluent. The retention order for metal cations was in good agreement with stability constants, except for cadmium, which was retained slightly stronger. The retention of transition metal cations decreased from pH 4.0 to 6.0 for both oxalate and citrate eluents. Such distinct behaviour is connected with two processes taking place in this chromatographic system with the increase of eluent pH. The elution strength of both citrate and oxalate eluents is increasing and the effective anion-exchange capacity decreases with the increase in the eluent pH, due to dissociation of carboxylic groups in both organic acids and in DDMAU.

5.2. Effect of eluent type on separation selectivity for metal cations

On-column complexation is responsible for the formation of negatively charged complexes and defines the retention of alkaline-earth and transition metal cations in the studied chromatographic system. It can be expected that both selectivity and column efficiency would be dependent on the type of complexing reagent used as the eluent.

The selectivity for transition metals was significantly higher in the case of the citrate eluent, due to differences in the conditional stability constants of the corresponding complexes. The degree of dissociation of the oxalic acid (pK_{a1} 1.20; pK_{a2} 4.20) is higher than that of citric acid (pK_{a1} 3.13; pK_{a2} 4.76), which means higher conditional stability constants for oxalates in the studied pH range and less selective

separation of different transition metals. In the case of citrate eluents non-complete formation of complexes means a difference in their effective negative charge, providing better ion-exchange selectivity, and improved resolution of adjacent peaks for the citrate eluent compared to the oxalate eluent (Fig. 5-1-1). It should be noted that both selectivity and resolution of chromatographic peaks are greater at lower pH values, where the differences in the effective charges of separated complexes are higher.

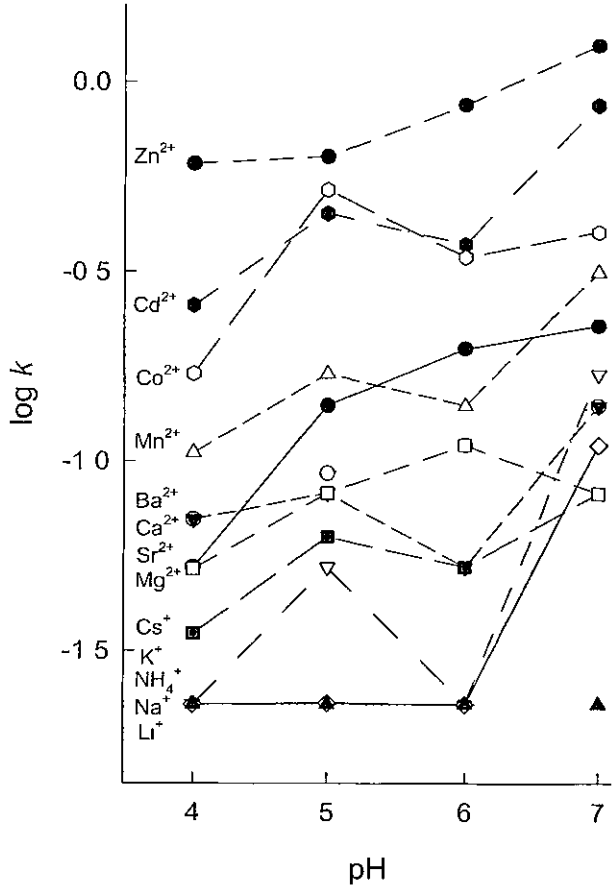

Figure 5-2-1. Dependence of retention factor for selected cations on eluent pH. Column Gemini C_{18}, coated with DDMAU, 100 x 4 6 mm I D. Eluent: 1 mM acetate buffer

To elucidate the role of on-column complexation in the retention of metal cations on a DDMAU coated zwitterionic stationary phase, the retention dependence on pH for an acetate buffer eluent was also obtained. It is known that acetate complexes with the selected cations are substantially weaker (Table 5-1-1) and thit should have an effect on both retention times and separation selectivity. Fig. 5-2-1 shows relatively weak retention of all studied cations in acetate eluents with small increases in retention at higher pH. This can be associated with the appearance of additional cation-exchange groups at the surface of ion-exchanger due to the dissociation of the carboxylic groups in the adsorbed DDMAU. The resulting retention order was typical for simple cation exchange chromatography:

$Li^+ \approx Na^+ \approx K^+ \approx NH_4^+ < Cs^+ < Mg^{2+} < Ca^{2+} \approx Sr^{2+} < Ba^{2+} < Mn^{2+} < Co^{2+} < Cd^{2+} < Zn^{2+}$.

However, the retention times of studied cations and selectivity values (Table 5-2-1) were too low (log $k < 0$) to obtain any reasonable separations with the acetate buffer as the eluent. Calculated selectivity values were higher for the citrate buffer as an eluent. With both, citrate buffer and oxalate buffer, the DDMAU modified column exhibited good selectivity for a number of species including Ba^{2+}/Mn^{2+}, Mn^{2+}/Co^{2+}, Co^{2+}/Cd^{2+}.

Table 5-2-1 Selectivity data for a range of inorganic cation pairs on Gemini C_{18} column coated with DDMAU Eluent 1 mM acetate buffer, pH range 4 0 to 7 0

pH	Na^+/Li^+	K^+/Na^+	NH_4^+/K^+	Cs^+/NH_4^+	Mg^{2+}/Cs^+	Ca^{2+}/Mg^{2+}
4.0	1.00	1.00	1.00	1.00	1.52	1.51
5.0	1.38	0.72	1.00	2.30	1.18	2.22
6.0	4.83	0.21	1.00	1.00	2.30	3.74
7.0	3.57	1.35	1.00	1.53	0.48	2.78
	Sr^{2+}/Ca^{2+}	Ba^{2+}/Sr^{2+}	Mn^{2+}/Ba^{2+}	Co^{2+}/Mn^{2+}	Cd^{2+}/Co^{2+}	Zn^{2+}/Cd^{2+}
4.0	1.32	1.00	1.51	1.62	1.52	2.36
5.0	1.00	1.13	2.07	2.65	1.16	1.41
6.0	1 00	2.64	1.64	2.47	1.08	2.33
7.0	1.00	1.00	2 26	1.28	1.12	1.44

5.3. Effect of eluent pH on separation efficiency

Column efficiency is an important factor in the separation of metal ions with on-column complexation. Kinetics of complexation depends upon the chemical nature of the ligands and on the pH of the eluent. To achieve maximum separation efficiency for on-column formed negatively charged complexes their stoichiometry should be minimal, preferably 1:1, and the coordination sphere of the central metal ion should not be fully saturated. Tridentate citric acid satisfies these demands forming 1:1 negatively charged complexes with the selected metal ions under acidic conditions. The other additional requirement for obtaining efficient separations is the absence of secondary equilibria, for example, the possible hydrolysis or formation of hydroxyl-complexes of transition metals. Both stoichiometry of the complexes and hydrolysis depends on pH of the eluent.

Tables 5-3-1 and 5-3-2 show the data on separation selectivity and efficiency calculated for metal cations separated on particle packed RP column (Gemini C_{18}) coated with DDMAU with citrate and oxalate buffers as eluents. The column efficiency

was almost twice higher for the citrate eluents, probably due to the necessity of a two-step on-column complexation reaction to achieve negatively charged oxalate complexes. It was shown that with a decrease in the eluent pH, both selectivity and efficiency increased for all cations (Figure 5-3-1).

However, inorganic anions are strongly retained with eluent pH less than 4.0. Therefore for all further experiments, eluents within the pH range from 4.0 to 6.0 were used to achieve the simultaneous separation of anions and cations. The efficiencies for the majority of cations were higher for a system with citrate as the eluent, so citrate buffer was used for the evaluation of the chromatographic performance of monolithic RP columns coated with DDMAU.

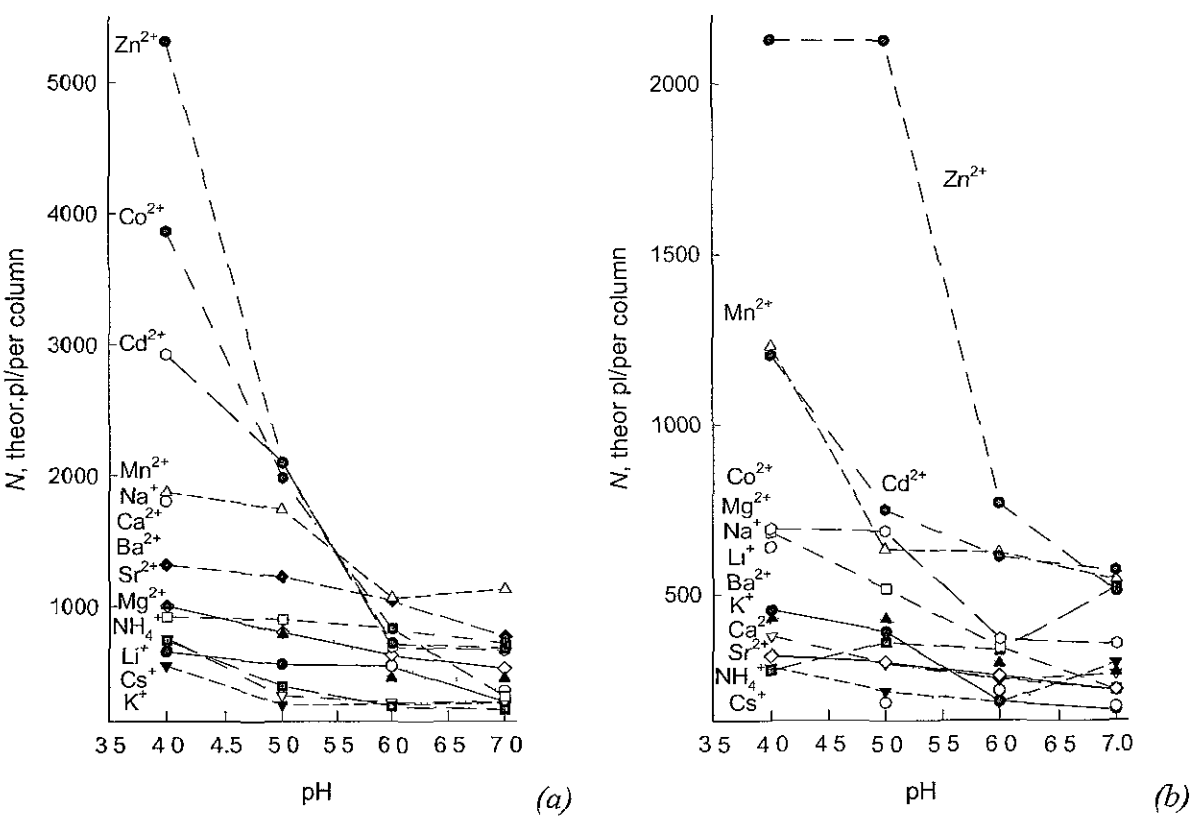

Figure 5-3-1. Column efficiency dependence on the eluent pH Gemini C_{18} column coated with DDMAU, 100 x 4 6 mm I.D. Eluent: 1 mM citrate buffer (a) and 1 mM oxalate buffer (b), F = 1.0 mL/min

Table 5-3-1. Selectivity and efficiency data for a range of inorganic cations on Gemini C_{18} column coated with DDMAU Eluent: 1 mM citrate buffer, pH range 4.0 to 7.0. Efficiency was calculated for the column length (100 mm)

pH	Cs^+		Na^+		K^+		NH_4^+		Li^+	
	N	α	N	α	N	α	N	α	N	
4.0	653	1.07	1799	1.08	545	1.77	755	1.06	737	
5.0	555	1.02	895	1.03	245	1.43	315	1.75	389	
6.0	543	1.06	542	1.00	250	1.06	262	1.71	233	
7.0	267	1.07	349	1.02	258	1.01	269	1.84	208	
	Ba^{2+}		Ca^{2+}		Sr^{2+}		Mg^{2+}			
	α	N	α	N	α	N	α	N		
4.0	2.93	1004	1.47	1316	1.12	1002	1.09	918		
5.0	2.77	787	1.02	1223	1.02	800	1.08	898		
6.0	2.45	449	1.01	1042	1.00	626	1.08	831		
7.0	1.31	446	1.01	765	1.00	522	1.07	310		
	Mn^{2+}		Co^{2+}		Zn^{2+}		Cd^{2+}			
	α	N	α	N	α	N	α	N		
4.0	2.60	1871	1.13	3863	1.68	5309	1.59	2923		
5.0	1.68	1739	1.09	1980	1.47	2093	1.03	2093		
6.0	1.04	1062	1.02	826	1.01	714	1.04	687		
7.0	1.03	1126	1.00	714	1.00	676	1.04	661		

Table 5-3-2. Selectivity and efficiency data for a range of inorganic cations on Gemini C_{18} column coated with DDMAU. Eluent: 1 mM oxalate buffer, pH range 4.0 to 7.0. Efficiency was calculated for the column length (100 mm)

pH	Li^+	Na^+		NH_4^+		K^+		Cs^+	
	N	α	*N*	α	*N*	α	*N*	α	*N*
4.0	454	1.11	638	1.00	286	1.49	378	1.00	276
5.0	391	1.24	183	1.05	216	1.56	298	1.03	360
6.0	185	1.07	216	1.12	182	1.49	249	1.05	335
7.0	162	1.56	169	1.19	298	1.02	266	1.01	522
	Mg^{2+}		Ca^{2+}		Sr^{2+}		Ba^{2+}		
	α	*N*	α	*N*	α	*N*	α	*N*	
4.0	1.92	682	1.33	322	1.03	319	1.09	429	
5.0	1.84	517	1.25	302	1.00	302	1.03	428	
6.0	1.69	343	1.10	252	1.00	261	1.26	296	
7.0	1.70	220	1.12	219	1.01	220	1.25	270	
	Mn^{2+}		Co^{2+}		Zn^{2+}		Cd^{2+}		
	α	*N*	α	*N*	α	*N*	α	*N*	
4 0	6.43	1230	1.07	1203	1.05	2128	1.00	692	
5.0	5.27	633	1.02	749	1.01	2128	1.01	686	
6.0	3.40	623	1.04	610	1.01	767	1.02	368	
7 0	2.64	543	1.01	570	1.02	508	1.03	354	

5.4. Evaluation of monolithic RP column coated with DDMAU

The use of the monolithic columns has many advantages, including the possibility of faster, more efficient separations at lower column backpressures and has already been shown to be suitable new phase for use in ion chromatography [35]. In this Section a monolithic RP column (Onyx C_{18} 100 x 4.6 mm I.D.) coated with DDMAU was evaluated and characterised. The retention of different metal cations was studied with a series of citrate eluents of concentration 1 m*M*, and pH between 3.25 and 6.0. Fig 5-4-1 shows the dependence of the retention of different alkali, alkaline-earth and transition metals cations as a function of pH with the 1 m*M* citrate buffer eluent. The obtained retention characteristics are similar to those observed earlier for the particle packed column coated with DDMAU.

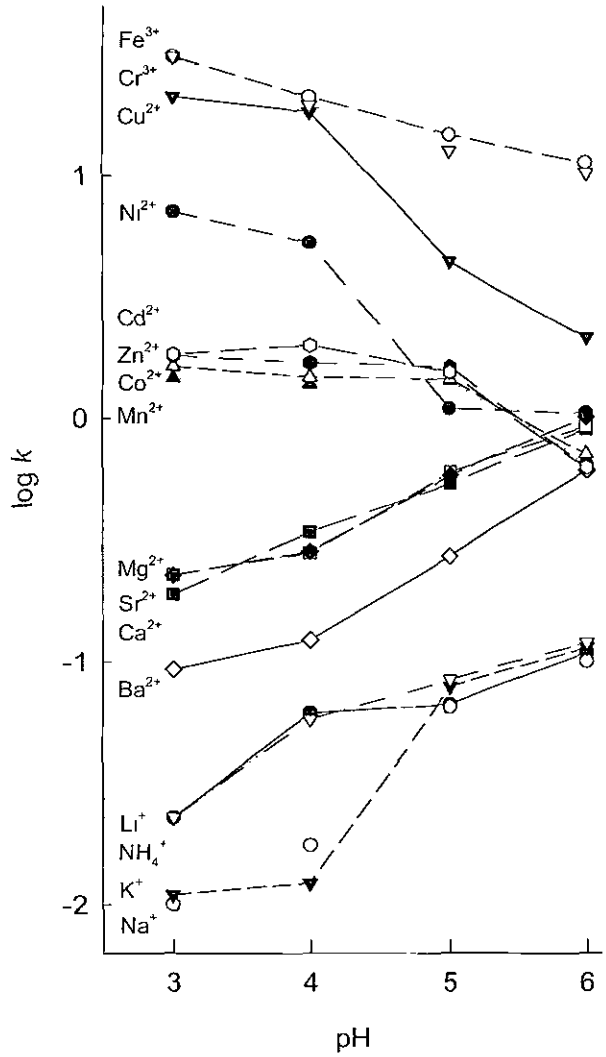

Figure 5-4-1 Dependence of retention factor for selected cations on eluent pH. Column: Onyx RP, coated with DDMAU, 100 x 3 0 mm I.D. Eluent: 1 mM citrate buffer

With a decrease in pH the retention increased for transition metal cations, which are able to form citrate complexes, while for alkali and alkaline-earth metal cations, with low stability constants for citrate complexes, an increase in retention with an increase in pH was noted. As for the particle packed RP column, a sharp increase in the retention for alkaline-earth metals and a sharp decrease in retention of transition metals were observed between pH 4.0 and 5.0. The variation of the citrate eluent pH caused more significant changes in retention for all cations on the monolithic RP column compared to the particle packed column. Also, citrate complexes of transition metals were less strongly retained on the monolithic RP DDMAU column than for the particle packed column. This could be connected to its lower cation exchange capacity. The retention order obtained for the monolithic RP DDMAU column with the citrate eluent coincides with the retention order observed for particle packed column:

$$Na^+ < K^+ < NH_4^+ < Li^+ < Ba^{2+} < Ca^{2+} \approx Sr^{2+} < Mg^{2+} < Mn^{2+} < Co^{2+} < Zn^{2+}\ Cd^{2+} < Ni^{2+} < Cu^{2+} < Cr^{3+} < Fe^{3+}.$$

In general, the selectivity of the monolithic RP DDMAU coated column under the conditions studied was slightly higher, while the efficiency of the 10 mm length column was also higher for the majority of metal ions studied.

A comparison of column performance under the same conditions using the citrate buffer as an eluent, showed that the maximum efficiency was higher for the monolithic RP DDMAU coated column, with a maximum of 61500 theoretical plates per meter, while for the particle packed RP column a maximum efficiency of 53100 theoretical plates per meter was achieved. The separation selectivity and resolution between the same analyte pairs was again higher for monolithic column (Tables 5-3-1 and 5-4-1). As a result, the monolithic column seems to be more suitable for the separation of cations, under these conditions.

The resolution of chromatographic peaks obtained with the monolithic RP DDMAU coated column with the citrate eluent was higher at lower pH, with resultant significant improvements in column efficiency (Fig. 5-4-2). As for the particle packed RP DDMAU coated column, the optimal eluent pH was found to be 4.0, giving the highest selectivity and efficiency values (Table 5-4-1).

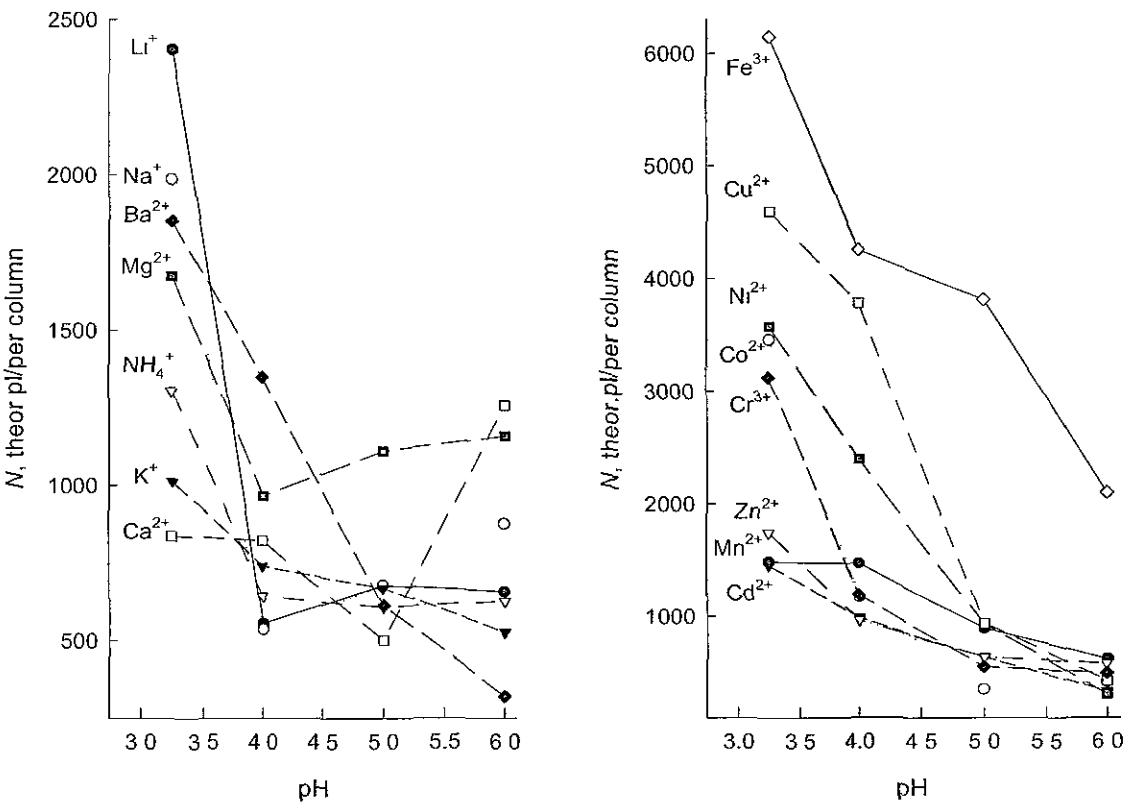

Figure 5-4-2. Column efficiency dependence on the eluent pH. Onyx C_{18} monolithic column coated with DDMAU, 100 x 3.0 mm I.D. Eluent 1 mM citrate buffer, F = 0.8 mL/min

Table 5-4-1 Selectivity and efficiency data for a range of inorganic cations on Onyx RP column coated with DDMAU Eluent 1 mM citrate buffer, pH range 3.25 to 6 0. Efficiency was calculated for the column length (100 mm)

pH	Na^+		K^+		NH_4^{2+}		Li^+	
	N		*α*	*N*	*α*	*N*	*α*	*N*
3.25	1986		2 00	1012	3.83	1304	3.80	2402
4.0	535		0.67	738	4.83	642	1.08	555
5.0	676		1.22	669	1.06	608	1.18	677
6.0	1076		1.14	526	1.04	626	1.06	657
	Ba^{2+}		Ca^{2+}		Sr^{2+}		Mg^{2+}	
	α	*N*	*α*	*N*	*α*	*N*	*α*	*N*
3.25	4.04	1852	2.45	836	1.01	754	1.21	1674
4.0	5.12	1347	2.29	820	1.01	1539	1.22	964
5.0	3.24	613	2.21	501	0.98	825	1.08	1111
6.0	2.16	320	1.51	1258	1.10	656	1.13	1159
	Mn^{2+}		Co^{2+}		Zn^{2+}		Cd^{2+}	
	α	*N*	*α*	*N*	*α*	*N*	*α*	*N*
3.25	5.81	1483	1.12	3463	1.16	1743	1.12	1449
4.0	4.04	1469	1.07	1171	1.01	963	1.01	985
5 0	2.64	897	1 02	356	1.00	642	1.12	639
6.0	0.75	625	1.04	421	1.05	585	1.06	338
	Ni^{2+}		Cu^{2+}		Cr^{3+}		Fe^{3+}	
	α	*N*	*α*	*N*	*α*	*N*	*α*	*N*
3 25	3.91	3579	5.43	4598	1.47	3124	1.00	6145
4.0	3.13	2397	2.14	3782	1.73	1186	1.01	4254
5 0	1.11	936	2.96	943	2.86	555	1.12	3815
6.0	1.07	310	2.05	431	4.16	498	1.11	2106

5.5. Investigation into the retention mechanism of cations on DDMAU-coated stationary phases

The retention of cations utilising on-column complexation is a complex mode of retention based upon the ability of the metal cations moving through the column bed in

the form of several different species. In the case of citric acid based eluents, with pH of the eluent between 4 and 6, and hypothetical divalent metal cations, these species may include the positively charged cation, M^{2+}, neutral protonated citrate complex, MHCit, and negatively charged complexes, $M(HCit)_2^{2-}$ Additionally, hydrolysis under weak acidic conditions should also be taken into consideration. Both negatively and positively charged species can interact with the adsorbed molecules of the zwitterionic, carboxybetaine type surfactant, DDMAU.

It was shown earlier in Section 5.2, that the use of less-complexing eluents (acetate buffer) resulted in weaker retention and the less selective separation of all metal cations, while the use of citrate and oxalate buffers as eluents resulted in stronger retention, selectivity and the relatively high resolution of transition metal cations. At the same time it was found that the citrate buffer is preferable as an eluent due to better overall resolution and higher efficiency. Taking into consideration that DDMAU coated columns exhibit mainly anion-exchange properties, this effect can be explained by the formation of more stable complexes with transition metal cations, than those formed with either oxalate or acetate. To prove this theory, the dependence of the retention of cations on the effective charge of the complexes formed was evaluated. To evaluate complex effective charge, the fractional diagrams of complex composition against pH were built using MEDUSA v.18 software (http://www.kemi.kth.se/medusa, Royal Institute of Technology, Stockholm, Sweden). From the resultant diagrams, which can be seen in Fig. 5-5-1, the fraction of each complex in the mixture and the total charge were calculated.

The resultant variation of complex effective overall charge with pH for the citrate buffer can be seen in Fig. 5-5-2, where the increase in retention with an increase of complex charge was observed. At pH 4.0 the complexes of cations of interest with citrate are predominantly positively charged, while only a small fraction of neutral or negatively charged complexes are present. With an increase in pH the fraction of negatively charged species grows and at pH 7.0 the majority of complexes are negatively charged. However, at pH 7.0 the surface of the stationary phase also has a negative charge, due to the dissociation of the terminal carboxylic acid groups of DDMAU, which increases the repulsion effects on the surface of the stationary phase and thus reduces the retention time of complexes. Furthermore, citrate further dissociates, so at pH 7.0 it is present in solution as the triple-charged anion (for citrate $pK_1 = 2.90$; $pK_2 = 4.35$; $pK_3 = 5.56$) and its effective elution strength increases, which means as a result the retention time for anions decreases.

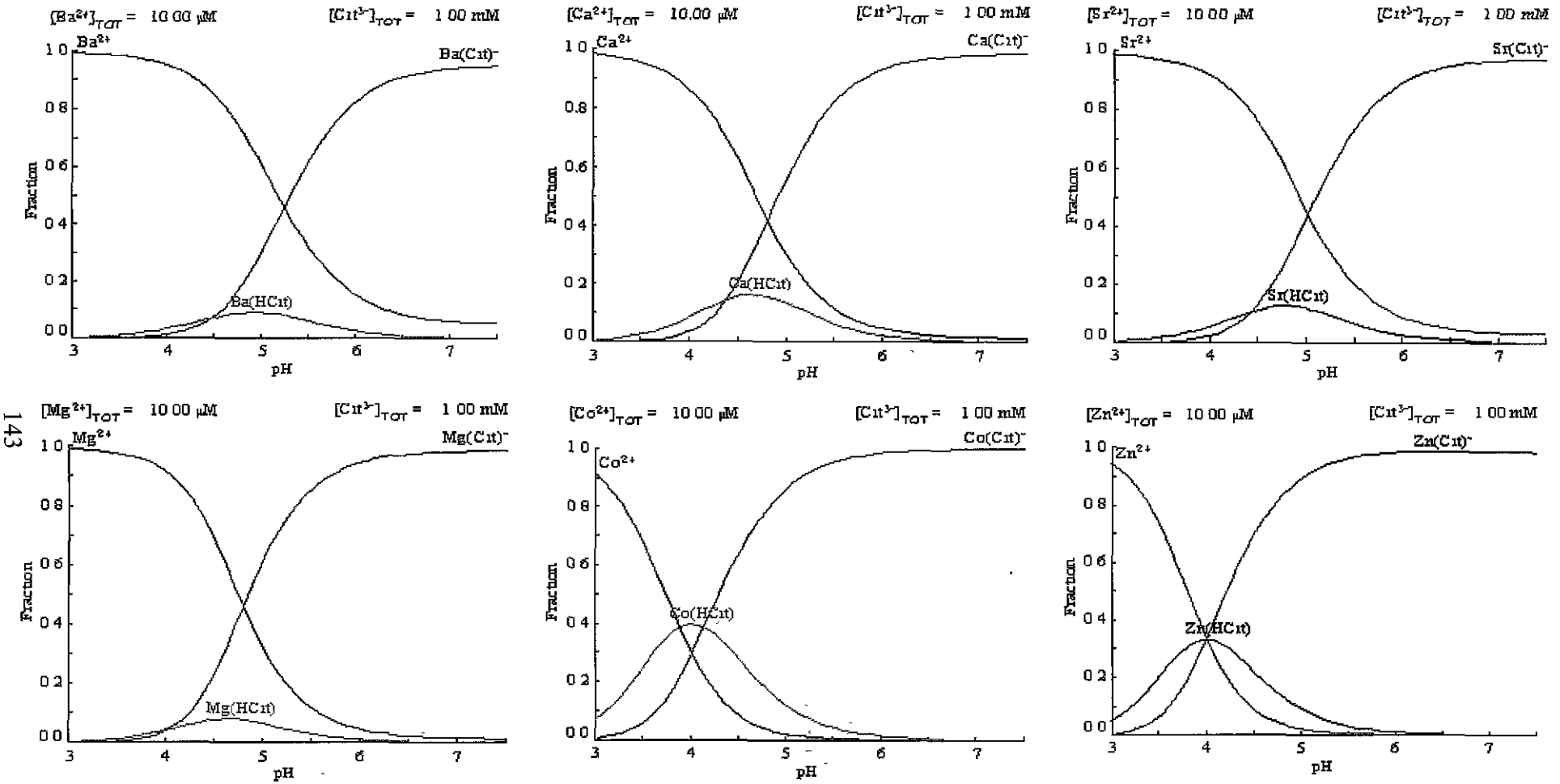

Figure 5-5-1. Equilibrium diagrams for metal complexes with citrate

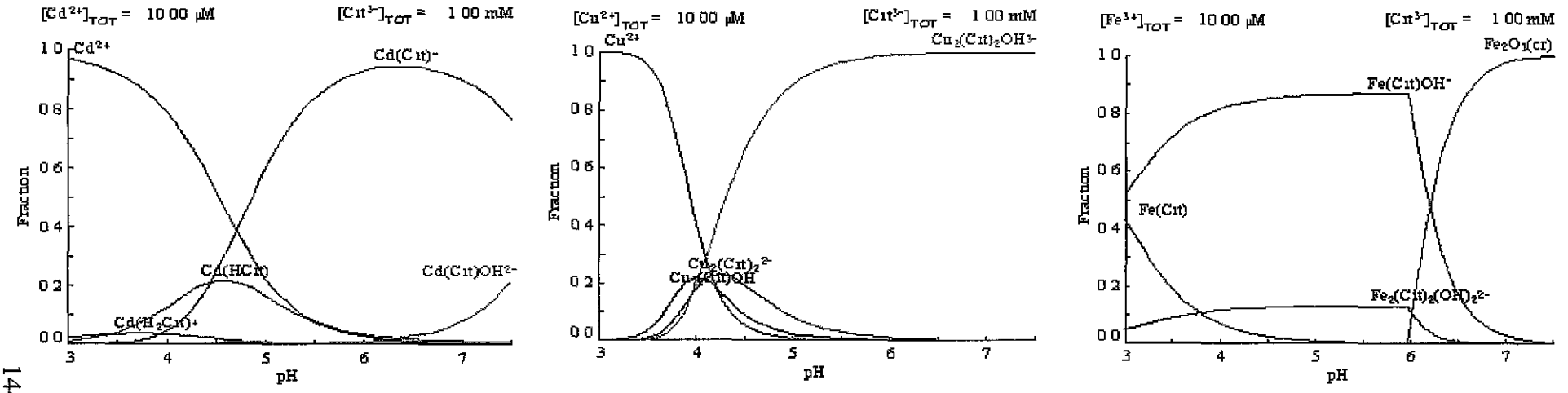

Figure 5-5-1. Equilibrium diagrams for metal complexes with citrate (end)

At the same time, an increase in retention for those highly stable metal complexes (Ni(II), Cu (II), Cr(III), Fe(III)) at low pH should be due to non-dissociation of carboxylic groups on the surface of the stationary phase and due to interactions between the ligand and DDMAU hydrophobic chains.

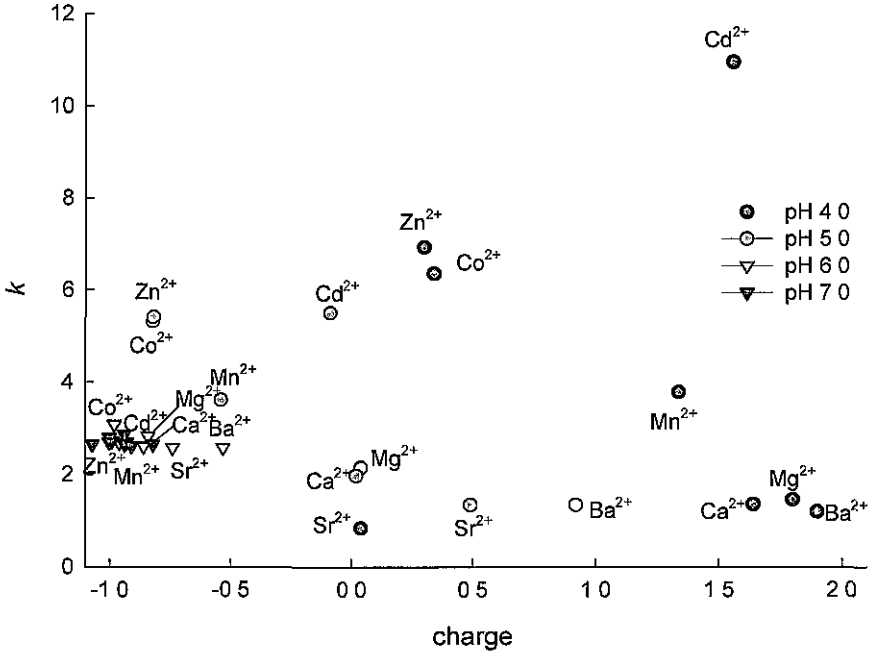

Figure 5-5-2. The dependence of capacity factor on the charge of metal complexes with citrate

5.6. Separation of metal cations

It was shown in previous Sections that DDMAU-coated stationary phases, can be potentially useful for the separation of different metal ions. Monolithic RP column coated with DDMAU revealed optimal efficiency for the separation of metal ions with an eluent of 1 m*M* citrate buffer, pH 4.0. The simultaneous separation of three alkaline-earth metal cations and chloride in less than 3 min on a Gemini C_{18} column coated with DDMAU is shown in Fig. 5-6-1. Chromatographic parameters of the separation are presented in Table 5-6-1.

Table 5-6-1 Chromatographic parameters for separation of standard cation mixture

cation	k	α	N (theor.plates per meter)	R_S
Ba^{2+}	0.76	-	10044	-
Ca^{2+}	2.11	2.77	13156	3.93
Mg^{2+}	2.35	1.11	10024	0.55

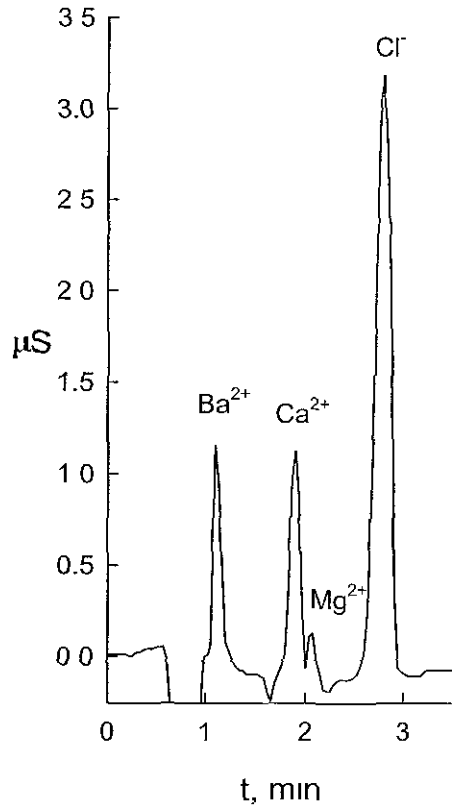

Figure 5-6-1 Chromatogram of model mixture of alkaline-earth cations and chloride. Column. Gemini C_{18} coated with DDMAU, 100 x 4.6 mm I.D., Eluent: 1 mM citrate buffer, pH 4.0 F = 1.0 mL/min

As the main advantage of zwitteriomc ion-exchangers is the possibility of simultaneous separations of cations and anions, it was decided to apply the DDMAU-coated stationary phases to this type of separation. Figure 5-6-2 shows the simultaneous separation of 5 cations and 6 anions in approximately 16 minutes.

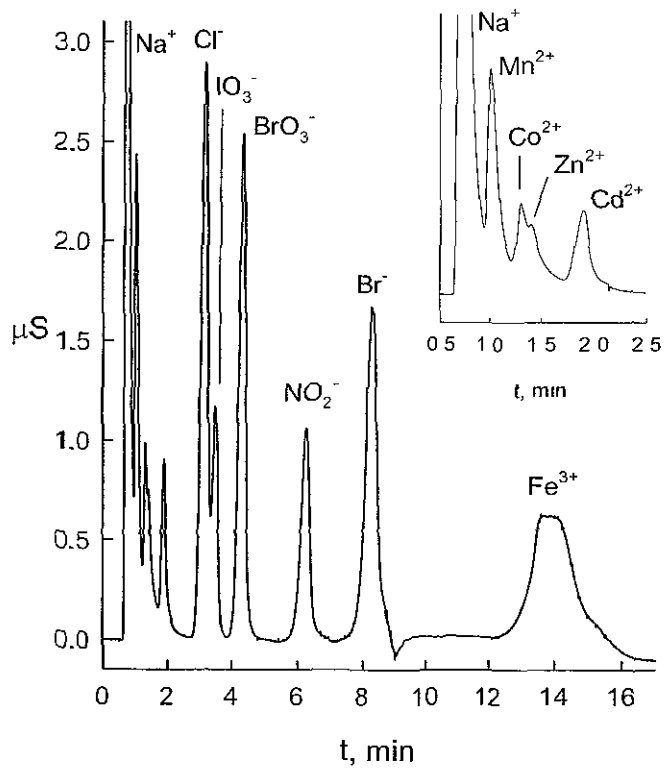

Figure 5-6-2. Chromatogram of simultaneous separation of cations and anions: sodium, manganese, cobalt, zinc, cadmium, chloride, iodate, bromate, nitrite, bromide and iron. Column: Gemini C_{18} coated with DDMAU, 100 x 4.6 mm I.D., Eluent: 1 mM citrate buffer, pH 4.0, F = 1 0 mL/min

Chromatographic parameters for this separation are presented in Table 5-6-2. It can be seen that peaks are not completely resolved and the efficiency could be improved. As expected the occurrence of on-column complexation in this chromatographic system has an affect upon separation efficiency for the negatively charged citrate complexes, compared to the efficiency calculated for peaks of inorganic anions. The calculated average efficiency was 15 % higher for inorganic anions than for metal complexes. Though peaks for some metal cations were not completely resolved, this chromatogram shows the possibility of the application of these columns to the simultaneous separation of anions and cations.

Table 5-6-2. Chromatographic parameters for separation of standard cation and anion mixture on Gemini C_{18} column coated with DDMAU (100 x 4.6 mm I.D.)

Ion	k	α	N (per meter)	R_S	Ion	k	α	N (per meter)	R_S
Mn^{2+}	2.33	-	18707	-	IO_3^-	6.77	1.13	11064	0.83
Co^{2+}	2.41	1.03	12768	0.18	BrO_3^-	8.65	1.28	17161	2.03
Zn^{2+}	2.71	1.12	11081	0.66	NO_2^-	12.88	1.49	15743	3.02
Cd^{2+}	4.47	1.64	10980	2.64	Br^-	17.24	1.34	16432	2.43
Cl^-	6.06	3.69	11886	5.39	Fe^{3+}	29.71	1.72	7797	2.83

The mixture of 10 cations and anions was also run on an Onyx RP column with 1 m*M* citrate buffer, pH 4.0 eluent. The separation, which can be seen in Fig. 5-6-3,was achieved in 35 minutes on a 20 cm long column. Chromatographic parameters for this separation can be seen in Table 5-6-3. All peaks, except the pair chloride/iodate were well resolved and the efficiency was slightly higher than for the Gemini C_{18} column.

Table 5-6-3. Chromatographic parameters for separation of standard cation and anion mixture on Onyx RP column coated with DDMAU (200 x 3.0 mm I.D)

Ion	k	α	N (per meter)	R_S	Ion	k	α	N (per meter)	R_S
Na^+	0.49	-	1385	-	BrO_3^-	9.17	1.34	11080	2.69
Mn^{2+}	1.83	3.73	22005	2.85	NO_2^-	14.07	1.53	10604	3.72
Cd^{2+}	2.83	1.55	14654	3.55	Br^-	18.67	1.33	12299	2.92
Cl^-	6.07	2.14	8655	4.75	Cu^{2+}	32.06	1.72	16969	5.91
IO_3^-	6.83	1.13	12643	1.26	Fe^{3+}	38.02	1.18	9258	1.60

Surprisingly the average column efficiency calculated for inorganic anions was this time only 60 % of that calculated for the separated citrate complexes of transition metal cations. This separation shows that monolithic RP columns coated with DDMAU can be used for the simultaneous separation and determination of transition metal cations and inorganic anions in different water samples.

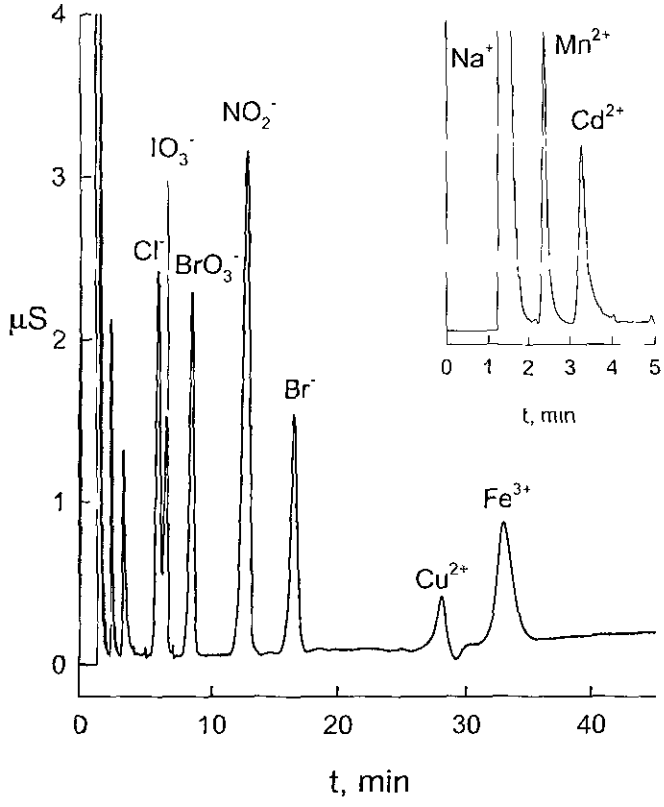

Figure 5-6-3 Chromatogram of simultaneous separation of cations and anions· sodium, manganese, cadmium, chloride, iodate, bromate, nitrite, bromide, copper and iron. Column. Onyx RP coated with DDMAU, 200 x 3.0 mm I.D., Eluent· 1 mM citrate buffer, pH 4.0, F = 0.8 mL/min

5.7. Application to the analysis of acid drainage water sample

The proposed method was applied to the simultaneous determination of anions and cations in a river water sample collected from an acid mine drainage stream (Avoca river, Co Wicklow, Ireland). The Avoca mines, from which the drainage stream originates, produced sulphuric acid and a variety of metals, including iron, copper, lead together with traces of silver and gold. Mining took place in a time when there were almost no environmental controls in place, and significant environmental degradation occurred in the area. This includes widespread contamination of the mine site and surrounding lands and a serious pollution of the Avoca river downstream of the mines.

Pyrite (iron sulphide) and chalcopyrite (copper iron sulphide) are the dominant minerals in Avoca mine area, with dissemination of sphalerite (zinc iron sulphide) and galena (lead sulphide). According to Gallagher *et al.* [36], approximately 2.5 million liters of the acid mine drainage was discharged each day into the Avoca river, adding

about 100 kg Zn, 10-15 kg Cu, 150 kg Fe, 1-2 kg Pb and 1600 kg sulphur per day. Sulphur was present in the drainage water samples as sulphate due to air oxidation.

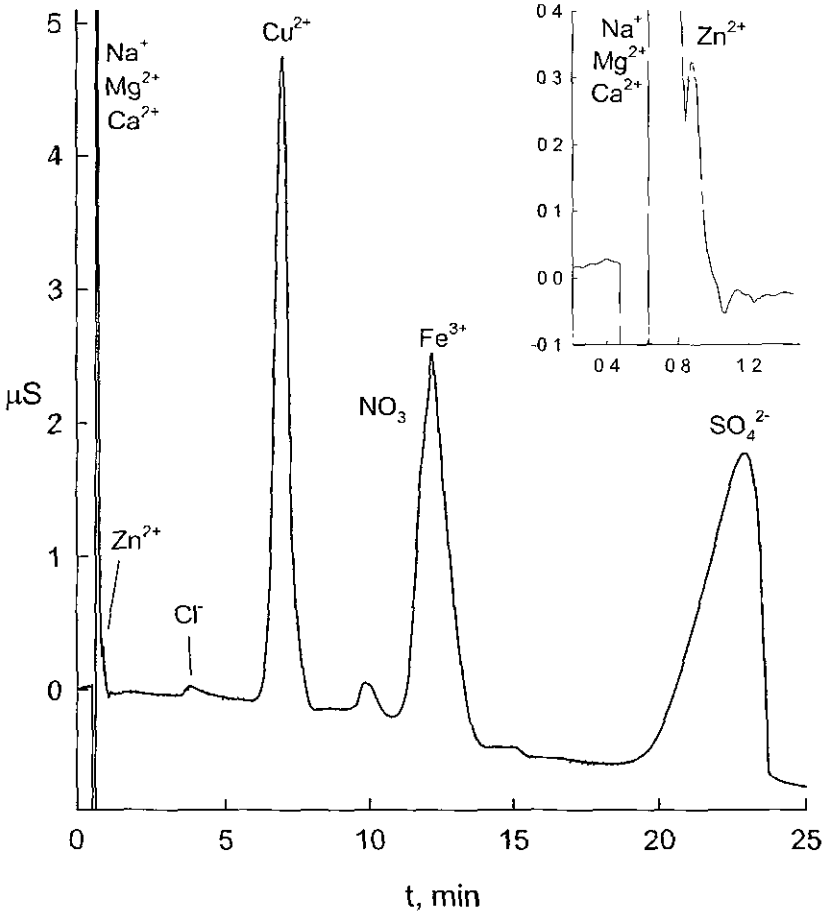

Figure 5-7-1. Chromatogram of an acid drainage water sample. Column: Onyx RP coated with DDMAU, 100 x 3.0 mm I.D., Eluent. 1 mM citrate buffer, pH 4.0, F = 0.8 mL/min

After the collection, the river water sample was treated with nitric acid for preservation. Prior the analysis the water sample was filtered through a 0.2 μm membrane filter and diluted 20 times with deionised water. To identify peaks, the sample was sequentially spiked with different cations and anions that were expected to be present in the sample. The chromatogram illustrating the simultaneous separation of anions (chloride, sulfate and added nitrate (co-eluted with Fe(III))) and cations (zinc, copper, iron, and co-eluted alkali and alkaline-earths) in natural water sample can be seen in Fig. 5-7-1.

5.8. Conclusions

The effect of eluent nature and pH on the separation of cations on Gemini C_{18} and Onyx RP columns coated with DDMAU was studied. The separation conditions on each column were optimised. An investigation into the mechanism of cation retention on these zwitterionic stationary phases was made. It was shown that with a citrate eluent

separation occurs based upon an anion-exchange mechanism, as both alkaline-earth and transition metals form anionic complexes. Using an oxalate eluent, separation occurs according to a cation-exchange mechanism for alkaline-earth metals, as they form 1:1 neutral complexes, and due to anion-exchange interactions for transition metals. Compared to pervious studies, which utilised polyaminocarboxylic complexones for the simultaneous separation of anions and cations, the improved selectivity was achieved when DDMAU coated columns and citrate eluents were used, with faster separations of more species in shorter times obtained, with no sample pre-treatment needed. The use of DDMAU coated columns with a citrate eluent allowed the simultaneous separation of up to 11 anions and cations in 16 to 35 minutes compared to 3 in 14 minutes achieved previously with a similar citrate eluent [27].

The retention order observed for these separations was different from those obtained by other authors:

$$Na^+ < Mn^{2+} < Co^{2+} < Zn^{2+} < Cd^{2+} < Cl^- < IO_3^- < NO_2^- < Br^- < Fe^{3+},$$

although, not all peaks were completely resolved (pairs Mn^{2+}/Co^{2+} and Co^{2+}/Zn^{2+}).

The obtained results for citrate eluent - DDMAU coated columns are comparable with results for the simultaneous separation of cations and anions on anion-exchange columns using polyaminocarboxylic complexones [12-16,18,21]. However, unfortunately in these previous studies there were no selectivity values presented. In earlier works, [12-14] the separation of anions and only calcium and magnesium was achieved using DCTA, EDTA, EGTA or DTPA as eluents. Schwedt *et al.* [15] obtained separation of nine anions and cations using a Nucleosil 10 – Anionen II column and a DCTA eluent in 20 min. The retention order was:

$$CH_3COO^- < Cl^- < NO_3^- < SO_4^{2-} < Ca^{2+} < Cd^{2+} < Mg^{2+} < Co^{2+} < Zn^{2+}.$$

However, all peaks were relatively broad and not completely resolved. Gautier *et al.* [16], using a Vydac 302 IC anion-exchange column and a DCTA eluent presented the separation of eight anions and cations in 25 min for which the retention order was:

$$Cl^- < NO_3^- < SO_4^{2-} < MoO_4^{2-} < Ca^{2+} < Hg^{2+} < Cu^{2+} < Co^{2+}.$$

All peaks for cations except cobalt (II) were poorly resolved and rather broad. Hajos *et al.* [15] achieved a separation of six anions and cation complexes with EDTA in less than 5 min utilising a Dionex AS9 column and a carbonate buffer eluent. The retention order on this columns was:

$$Cl^- < Mn^{2+} < Cd^{2+} < Al^{3+} < Ni^{2+} < Co^{2+}.$$

All peaks were completely resolved. Lucy *et al.* [18] utilised a Delta-Pak C_{18} RP-column coated with hexadecyltrimethylammonium bromide and a phosphate buffer as

eluent. Using this system he obtained the separation of four metal cation complexes with EDTA in 20 min. The retention order obtained on this column was:

$$Pb^{2+} < Cd^{2+} < Cu^{2+} < Zn^{2+}.$$

One of the disadvantages of all the methods described above was the need for preliminary sample treatment with the various complexones. As well in the majority of cases, peaks were not completely resolved and broad.

The results obtained for DDMAU coated column were also compared with results published by Haddad *et al* [27], where the possibility of the simultaneous separation of transition metal cations and nitrate was demonstrated using a citrate-oxalate eluent. The retention order on a 25 cm long Vydac 302 IC anion-exchange column was reported to be the same for citrate, oxalate, and citrate-oxalate eluents:

$$Mn^{2+} < Cd^{2+} < Pb^{2+} < NO_3^- < Zn^{2+} < Co^{2+} < Cu^{2+} < Ni^{2+}.$$

The retention order observed for the same group of metal cations with a citrate buffer on the DDMAU coated columns was slightly different:

$$Mn^{2+} < Co^{2+} < Zn^{2+} < Cd^{2+} < Ni^{2+} < Cu^{2+} < Cr^{3+} < Fe^{3+} \approx NO_3^-.$$

Unfortunately, no selectivity values were presented in [27], but it was shown that with the 20 m*M* citrate eluent, pH 4.5, copper (II) was retained for 25 minutes. Authors reported that the use of the oxalate eluent resulted in a shorter run time, but citrate eluent gave better sensitivity, so this was the reason for the use of a mixed citrate-oxalate eluent for the separation of nitrate, lead (II), and copper (II), with the separation performed in approximately 17 minutes.

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CHAPTER 6. SEPARATION OF NUCLEIC ACIDS PRECURSORS

Nucleotide, nucleoside and nucleobase analysis is essential in many biomedical and clinical fields due to their essential role in cellular metabolism. Therefore, a variety of separation options exhibiting various selectivities is important in analytical biochemistry. Several analytical methods to determine nucleotides, nucleosides and their bases in a variety of matrices have been proposed, with the use of RP HPLC being often reported [1-18]. However, for charged molecules the use of RP chromatography can result in both poor selectivity and insufficient retention and resolution. RP ion-pairing chromatography is an approach applied to address the above difficulties, and has exhibited impressive selectivity and efficiency for the separation of such charged species [7-11,13,14,19,20]. Ion-exchange chromatography offers a potential alternative, however recent studies show improvements can still be made in terms of efficiency, resolution and overall retention times when trying to separate a diverse range of nucleic acid precursors [6,21,22]. Other modes of chromatography for the separation of nucleotides, utilising normal phase columns [23], moleculary imprinted stationary phases [24], and zwitterionic immobilised artificial membrane columns [12] have also been reported. Recently a few works using amphoteric and zwitterionic sulphobetaine and betaine type surfactants to modify particle packed reversed-phase substrates have been conducted [25,26]. In these studies it was suggested that separation occurs mainly due to the analyte electrostatic interaction with the surface of the modified RP substrate, combined with residual hydrophobic interactions with native unmodified RP [26]. However, in these preliminary studies the separation of only nucleosides and nucleobases was explored.

Due to the zwitterionic nature of the DDMAU stationary phase, and the existence of nucleobases and nucleosides in either cationic or neutral form, and nucleotides in anionic form over a wide pH-range, the possibility of the simultaneous separation of these analytes on the surfactant modified phases was investigated. The possibility of utilising a combined eluent pH, concentration, and flow rate gradient for this complex mixture of nucleic acid precursors was investigated. The results presented in the following Chapter demonstrate this possibility with the simultaneous separation of nucleobases, nucleosides and nucleotides, existing in cationic, neutral and anionic forms on RP columns coated with the amphoteric carboxybetaine-type surfactant.

6.1. Nucleobase, nucleoside and nucleotide selectivity study

For the investigation of nucleic acid precursor selectivity on Gemini C_{18} and Chromolith RP stationary phases modified with DDMAU the retention dependence on pH and ionic strength of the eluent were studied Ammonium acetate buffer solution was chosen as the eluent for this study due to its compatibility with both UV-absorbance (at the chosen wavelength) and electrospray ionisation mass spectrometric (ESI-MS) detection. In this study in was decided to use only DDMAU as a stationary phase modifier due to the low stability of DDMAB and the need to constantly add the surfactant to the eluent.

6.1.1 The effect of eluent pH on the retention of analytes

It has been previously demonstrated in Chapter 3 that the weak acid terminal group of DDMAU exhibits strong affinity to H^+, and that the eluent pH can be used to control the degree of ionisation of the carboxylic acid functionality. This in turn acts to shield/de-shield the internal quarternary anion exchange site of the molecule and has a considerable effect upon the retention of anionic species. The carboxylic acid group of DDMAU has a pKa value of ~ 5.0. Using eluent conditions where pH < 5.0, the overall charge of the absorbed molecule is increased, which facilitates increased attraction of anionic species for the quaternary ammonium site of the zwitterionic molecule. Increasing the pH to pH > 5.0 has the opposite effect, reducing the retention of anions, allowing shorter chromatographic runs.

The effect of eluent pH on the retention of nucleic acids precursors was investigated here using a 1 m*M* ammonium acetate eluent over the pH interval 4.6 to 7.0, under which conditions the adsorbed molecule should be predominantly in its zwitterionic form.

Nucleobases. The nucleobases exhibit least retention of all the precursors investigated due to their lack of charge under non-acidic conditions. From Fig. 6-1-1 it can be observed that there was no significant change in retention over this pH range for selected nucleobases, except cytosine. Of the five species cytosine has the highest pK_a (Table 6-1-1), and so at pH values approaching 4.5, there would be simultaneous protonation of the terminal weak acid group of the absorbed DDMAU and an increased repulsion from the strong quaternary ammonium site, leading to the decrease in retention shown.

Table 6-1-1. pK_{ab} and pK_{aa} values for nucleotides, nucleosides and nucleobases [27]

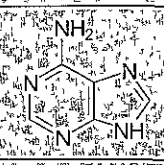
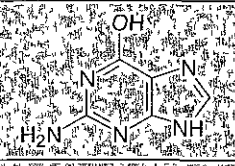
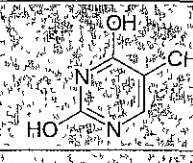
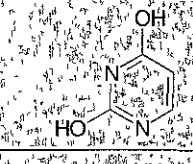
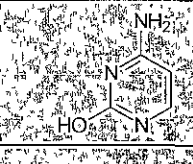
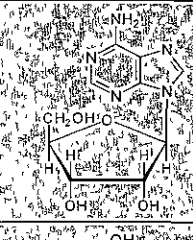
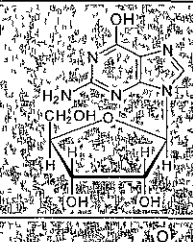
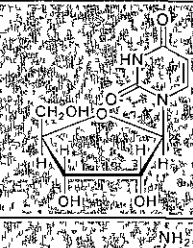
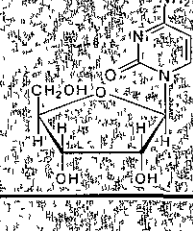
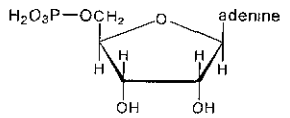
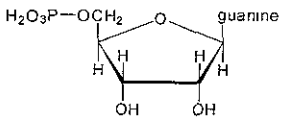
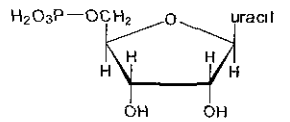
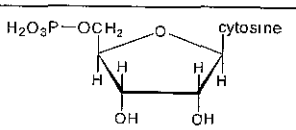
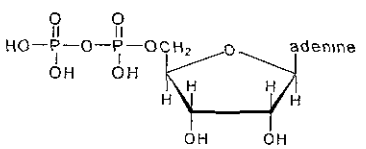
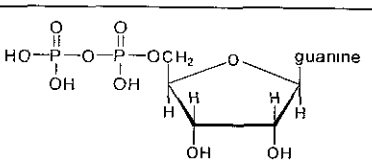
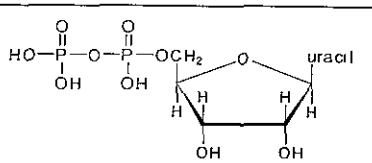
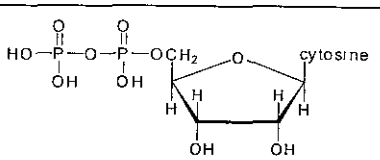
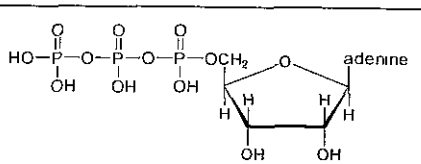
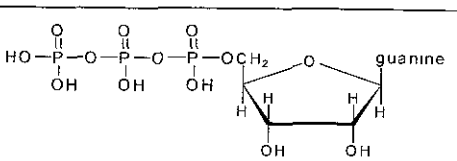
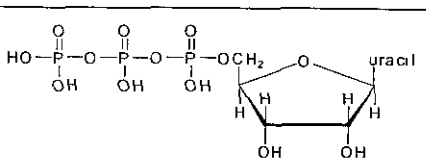
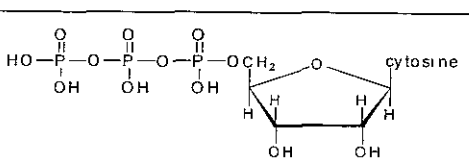
			pK_{ab}	pK_{aa1}	pK_{aa2}
		Nucleobases			
Ade	Adenine		4.1	9.8	-
Gua	Guanine		3.3	9.2	12.3
Thy	Thymine		~0	9.9	13.0
Ura	Uracil		~0.5	9.5	13.0
Cyt	Cytosine		4.4	12.3	
		Nucleosides			
A	Adenosine		3.3	6.2	12.5
G	Guanosine		1.6	9.2	12.4
U	Uridine			9.2	12.5
C	Cytidine		4.2	-	12.5

Table 6-1-1.(continuation)

Nucleotides					
AMP	Adenosine-5'-monophosphate		3.8	-	6.2-6.4
GMP	Guanosine-5'-monophosphate		2.4	9.4	6.1
UMP	Uridine-5'-monophosphate		~ 1.0	9.4	6.4
CMP	Cytidine-5'-monophosphate		4.4	-	6.3
ADP	Adenosine-5'-diphosphate		3.9	-	6.1-6.7
GDP	Guanosine-5'-diphosphate		2.9	9.6	6.3
UDP	Uridine-5'-diphosphate		~ 1.0	9.4	6.5
CDP	Cytidine-5'-diphosphate		4.6	-	6.4
ATP	Adenosine-5'-triphosphate		4.0	-	6.0-7.0
GTP	Guanosine-5'-triphosphate		3.3	9.3	6.3
UTP	Uridine-5'-triphosphate		~ 1.0	9.6	6.6
CTP	Cytidine-5'-triphosphate		4.8	-	6.6

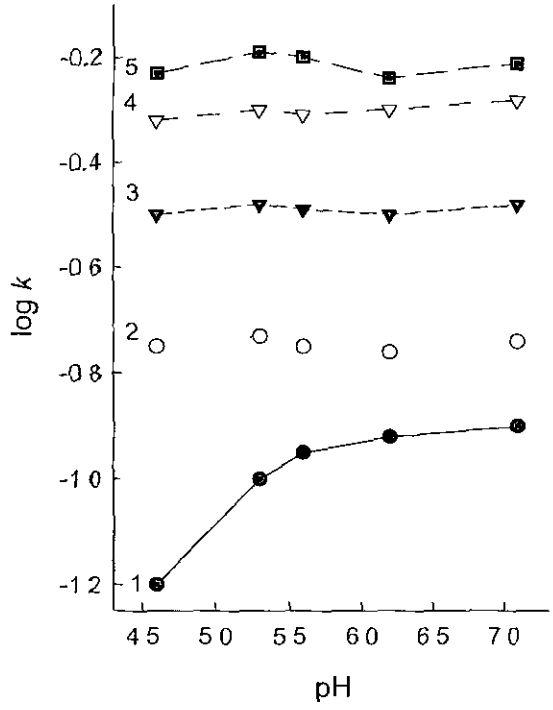


Figure 6-1-1. Dependence of retention factor for 1 - cytosine, 2 - uracil, 3 - thymine, 4 - guanine, 5 - adenine on eluent pH.
Column: Chromolith RP, 100 x 4.6 mm I.D., coated with DDMAU.
Eluent: 1 mM ammonium acetate buffer

Nucleosides The retention behavior of the nucleosides is also related to their pKa values (Fig. 6-1-2). Thus, guanosine and uridine are largely neutral in the pH range of the present study, and therefore do not experience a significant change in retention with an increase of the eluent pH. However, adenosine and cytidine, which are protonated at pH < 3.3 and 4.2 respectively, behave similarly to cytosine above under acidic eluent conditions.

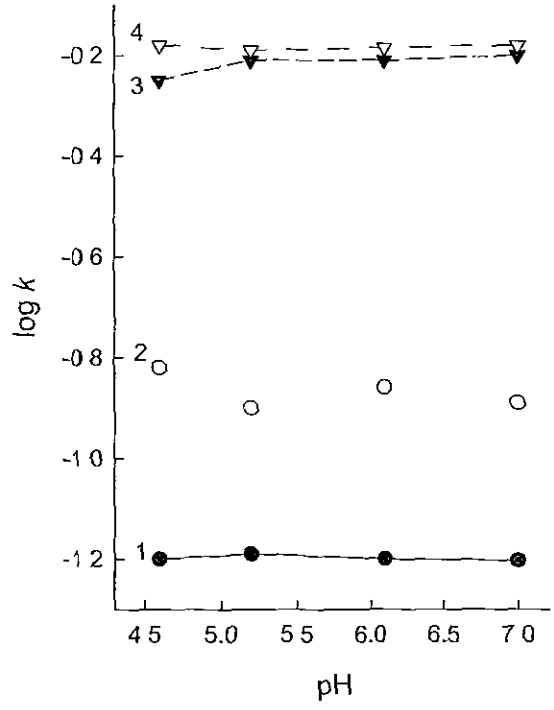


Figure 6-1-2 Dependence of retention factor for 1 - cytidine, 2 - uridine, 3 – adenosine, 4 - guanosine on eluent pH
Column: Chromolith RP, 100 x 4.6 mm I.D., coated with DDMAU.
Eluent: 1 mM ammonium acetate buffer

Using a 5 m*M* phosphate buffer eluent the effect of reducing pH below 4.6 was briefly investigated. At pH 7, the eluent order for nine nucleobases and nucleoside on a 100 x 4.6 mm DDMAU coated monolith was guanosine > adenosine = adenine > guanine > thymine > uracil > cytidine = uridine > cytosine. At pH 5.3 this selectivity was found to be the same, whilst at pH 3.2, the following was observed: guanosine >> thymine > uridine > adenosine > guanine = uracil > adenine > cytidine = cytosine. As mentioned above the selective reduction in the retention of the more cationic species was quite clear.

Figure 6-1-3 shows an optimised separation of a nucleoside and nucleobase mixture on a DDMAU modified monolith. To separate 8 nucleic acids precursors as shown, a 20 cm long DDMAU column was used (2 coupled 100 x 4.6 I.D. mm columns). With the longer column, to reduce retention of the cationic species, a 3 m*M* acetic acid eluent (pH 3.7) was utilised, combined with a flow gradient elution to further reduce the overall analysis time (0.0 min to 6.0 min – 0.6 mL/min; 6.0 min to 10.0 min – 0.6 mL/min to 1.4mL/min).

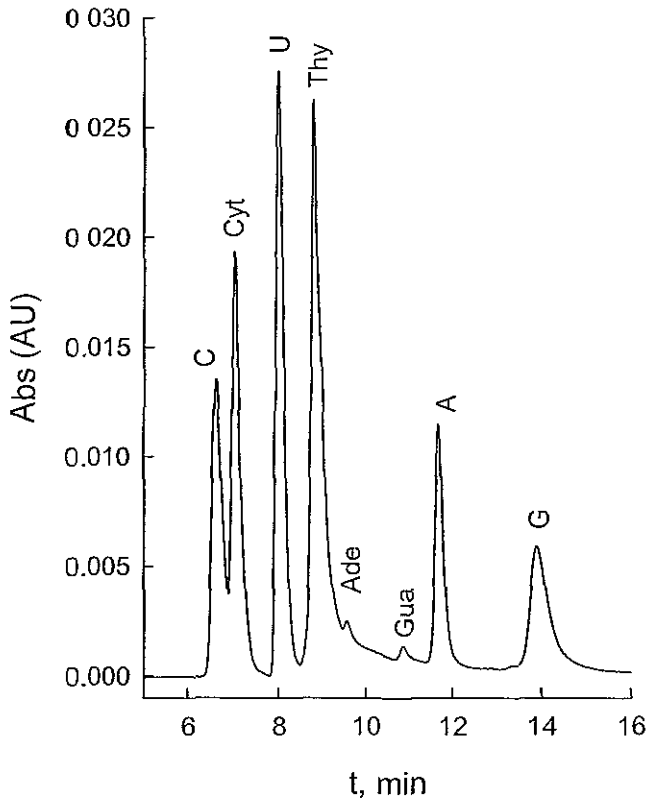


Figure 6-1-3. Chromatogram of nucleoside and nucleobase mixture. Mobile phase: 3 mM acetic acid, pH 3.7. Flow gradient elution: min to 6.0 min – 0.6 mL/min; 6.0 min to 10.0 min – 0.6 mL/min to 1.4 mL/min Column: Chromolith RP, 200 x 4.6 mm I.D., coated with DDMAU. Column was thermostated at 8 5 °C Direct UV detection at 260 nm

Nucleotides. Contrary to nucleosides and their corresponding bases, nucleotides are predominantly negatively charged over the pH range of this study due to their low first pK_{a1} for attached phosphate groups, the second pK_{a2} being approximately 6.5 (Table 6-1-1). The charge on the nucleotide increases with the increasing number of phosphate groups present, i.e nucleotide mono- < di- < tri- phosphate. Therefore, the separation of nucleotides at this pH range of study occurs due to anion exchange. Increasing the pH of the eluent from between 4.0 and 6.0 results in an overall decrease in positive charge on the modified stationary phase, whilst the charge upon the nucleotides remains relatively unchanged, and as the result the retention time of nucleotides decreased with increasing pH (Fig. 6-1-4).

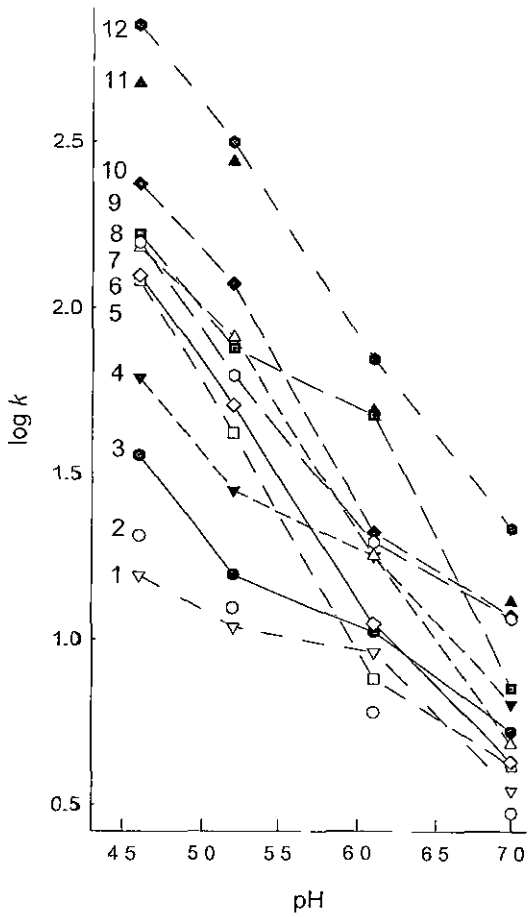


Figure 6-1-4. Dependence of retention factor for 1 – UMP, 2 - CMP, 3 – AMP, 4 – GMP, 5 – CDP, 6 – UDP, 7 – CTP, 8 – UTP, 9 - ADP, 10 – GDP, 11 – ATP, 12 – GTP on eluent pH.
Column: Chromolith RP, 100 x 4.6 mm I.D , coated with DDMAU
Eluent. 5 mM ammonium acetate.

This effect allowed a pH gradient approach to be explored, with retention of strongly retained anionic species reduced whilst weakly retained neutral species remain unaffected (Section 3.3.3).

6 1 2. The effect of eluent concentration on the retention of analytes

For the effect of eluent concentration on the retention of nucleosides and nucleobases (adenine, guanine, thymine, cytosine, uracil, adenosine, guanosine, cytidine

and uridine), a series of eluents of different concentrations ranging from 0.1 to 10 mM, each at pH 4.6, was prepared.

The effect of altering the concentration of the ammonium acetate eluent on retention using a 10 cm DDMAU monolith is shown in Fig 6-1-5. A decrease in retention was observed with an increase in eluent concentration for all of the above species, however considerable differences in observed slopes were recorded.

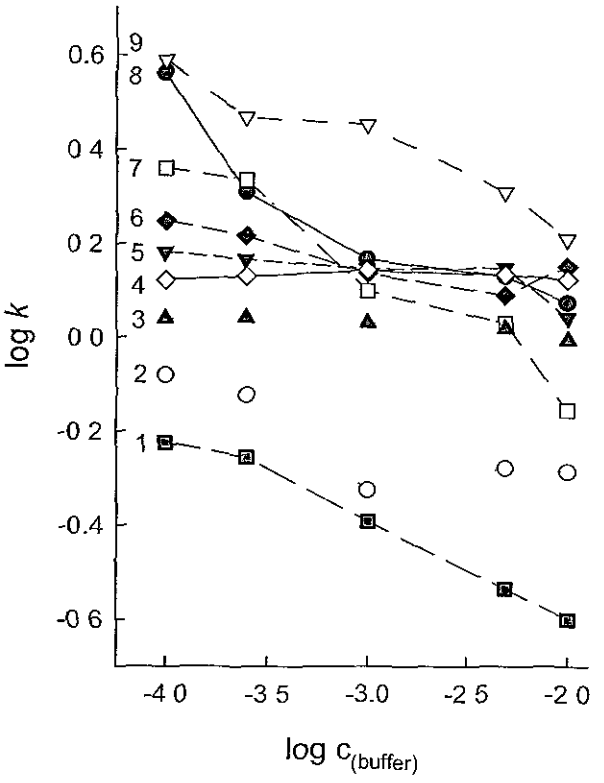


Figure 6-1-5. Dependence of retention factor on concentration of eluent (ammonium acetate buffer, M) for 1 - cytosine, 2 - cytidine, 3 - uracil, 4 - guanine, 5 - uridine, 6 - thymine, 7 - adenine, 8 - adenosine, 9 – guanosine Column: Chromolith RP, 100 x 4 6 mm I.D., coated with DDMAU

Applying a linear regression model to the data shown resulted in correlation coefficients for each analyte of $R^2 = 0.989$ for cytosine (slope = - 0.196), $R^2 = 0.972$ for adenine (slope = -0.248), $R^2 = 0.945$ for adenosine (slope = -0.213), $R^2 = 0.943$ for guanosine (slope = -0.170), $R^2 = 0.882$ for cytidine (slope = -0.107), $R^2 = 0.837$ for uracil (slope = -0.021), $R^2 = 0.766$ for thymine (slope = -0.063), $R^2 = 0.654$ for uridine (slope = -0.052), and $R^2 = 0.517$ for guanine (slope = -0.307). From the above it can be seen that more linear responses were seen for those species exhibiting some cationic character at this eluent pH, including cytosine, adenine and adenosine (see Table 6-1-1), indicating a dominant ion exchange retention mechanism for these species. As expected, for the remaining predominantly neutral species, less linear behaviour was observed, with hydrophobic interactions dominating retention. At this eluent pH the optimal ammonium acetate concentration for overall resolution was found to be 0.25 m*M*.

6.2. Effect of Ca^{2+} additives to the eluent on the retention of nucleotides

Ammonium acetate is a relatively weak eluent for separations of highly charged anions, so an eluent concentration of up to 100 m*M* was required for the elution of all nucleotides. Even using an eluent of such strength, the triphosphates were retained for approximately 90 min on the modified monolithic phase. It is known that adding calcium or magnesium salts to the eluent can reduce the retention of nucleotides [28] due to complexation with the negatively charged phosphate groups, thus reducing the overall negative charge of the molecules and therefore their retention due to anion exchange interactions. In this work, calcium salts were added to evaluate this effect. Figure 6-2-1 shows that the variation in retention observed for 12 nucleotides when increasing amounts of $CaCl_2$ was added to the ammonium acetate eluent. The addition of 1 m*M* of $CaCl_2$ reduces the retention time of strongly retained nucleotides by up 10-15 times. However, although such a strong effect was observed, it was also found that the use of calcium salts in this method led directly to reduced column lifetimes due to column blockages resulting from calcium induced precipitation and so was not used further.

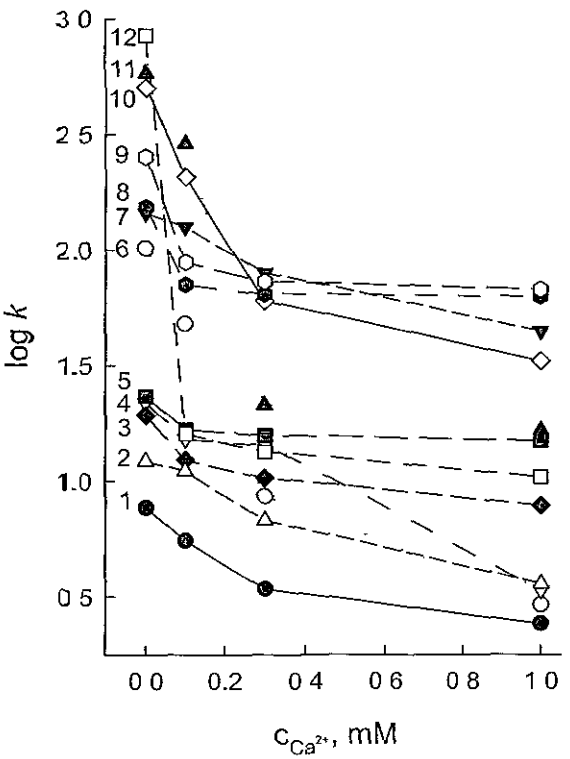


Figure 6-2-1. Dependence of retention factor on eleunt concentration of $CaCl_2$ added to 5 mM ammonium acetate solution for 1 – CMP, 2 – UMP, 3 – AMP, 4 – CDP, 5 – GMP, 6 – UDP, 7 – CTP, 8 – GDP, 9 – ADP, 10 – UTP, 11 – ATP, 12 – GTP. Eluent 5 mM ammonium acetate. Column: Chromolith RP, 100 x 4.6 mm I.D., coated with DDMAU

6.3. Separation of nucleic acids precursors using a novel triple gradient approach

The combination of the monolithic substrate, which allowed the application of elevated flow rates, and the DDMAU coating, which was zwitterionic under the

conditions used and showed a considerable pH dependent selectivity, meant the application of a combined flow, and eluent concentration and pH gradient could be demonstrated here for the separation of these charge diverse species (Figure 6-3-1). An eluent gradient from 5 mM acetic acid, pH 3.6 to 100 m*M* ammonium acetate, pH 5.3 was applied, initially delivered at 0.5 mL/min then increased up to 4.5 mL/min by the end of the run. Column temperature was maintained at 8.5°C, as the decrease in temperature resulted in slight increases in the retention time of nucleosides and their bases, which had the effect of small improvements in overall selectivity.

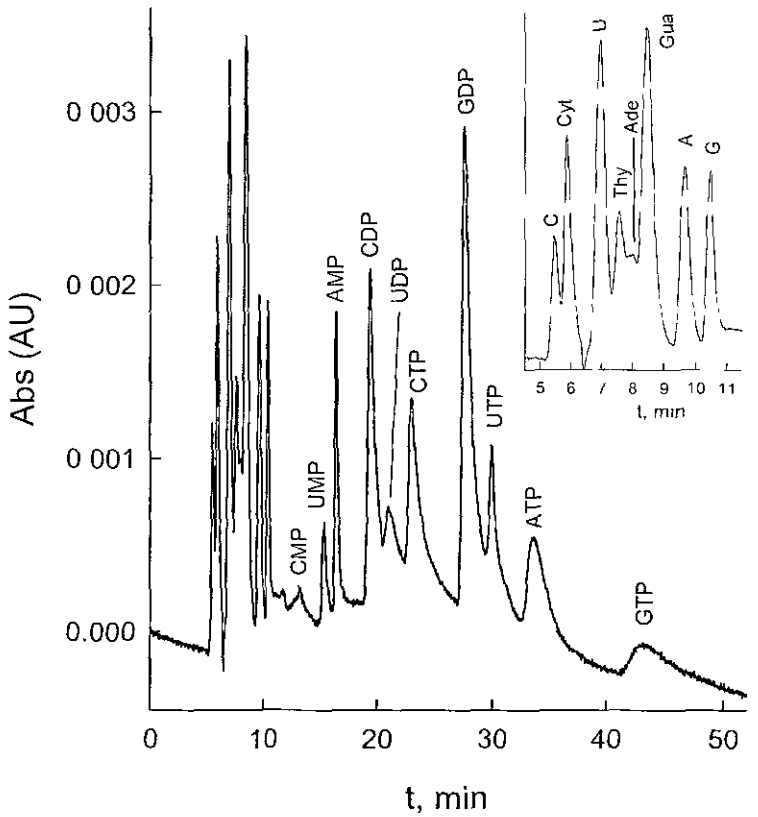


Figure 6-3-1. Separation of standard mixture of 10 nucleotides, 4 nucleosides and 4 nucleobases with eluent concentration/pH/flow rate triple gradient applied Column: Chromolith RP coated with DDMAU 200 x 4.6 mm. Eluent A: 5 mM acetic acid, pH 3.6. Eluent B: 100 mM ammonium acetate, pH 5.3.

Gradient. 0 0 - 8.0 min – 100 % A, F = 0.5 mL/min,

8.0 - 14.0 min – 100 % A to 50 % A – 50 % B, F = 0 5 to F = 2 0 mL/min,

14.0 - 22 0 min – 50 % A – 50 % B to 100 % B, F = 2.0 mL/min;

22 0 - 28.0 min –100 % B, F – 2 0 to 3.5 mL/min,

28.0 - 32.0 min – 100 % B, F = 3.5 to 4.5 mL/min;

32.0 - 50.0 min – 100 % B; F = 4.5 mL/min.

Column was thermostated at 8 5 °C Direct UV detection at 260 nm

Under these starting conditions, the standard mixture of nucleotides, nucleosides and bases contains compounds in cationic (+1), anionic (-1, -2, -3) and neutral form. Figure 6-3-1 shows the resultant separation, with cationic species eluting first (C, Cyt), followed by neutrals (U, Thy, Ade, Gua, A, G), followed by anions, - 1s (CMP, UMP, AMP), - 2s (CDP, UDP, GDP) and – 3s (CTP, UTP, ATP and GTP). Inset shows the expanded view of the closely eluting nucleobases and nucleosides in the above mixture.

The above triple gradient method and column was applied to the analysis of a variety of commercial yeastolate samples for nucleobase, nucleoside and nucleotide content (Figure. 6-3-2).

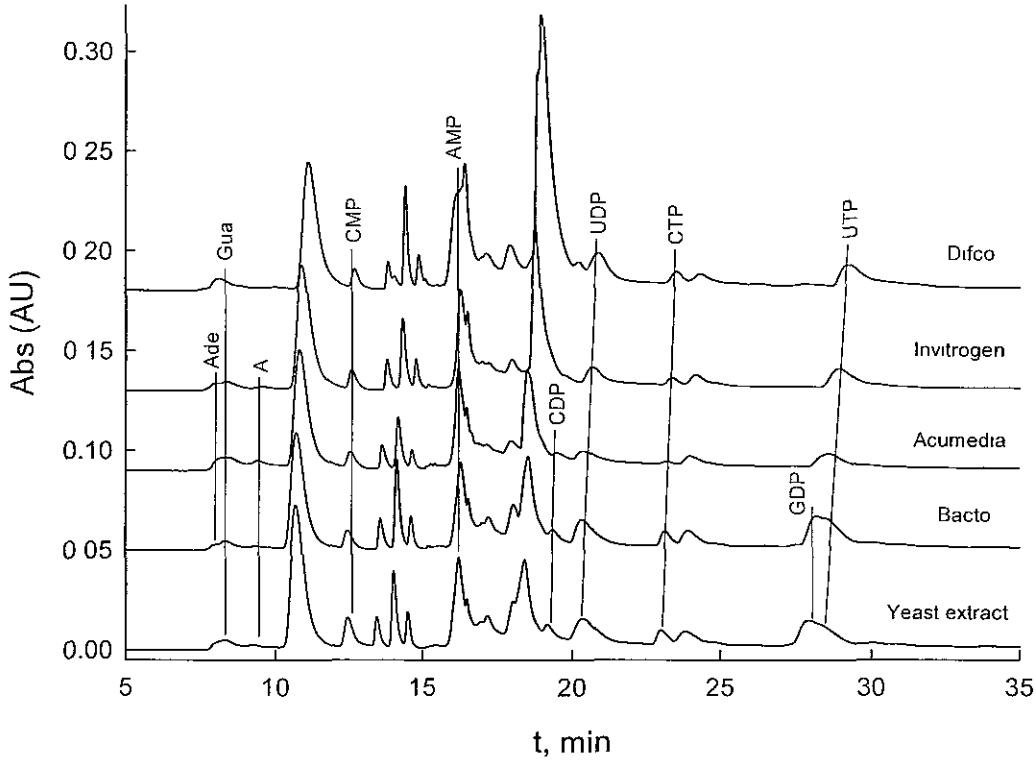


Figure 6-3-2. Chromatograms of commercial yeastolate extract samples using triple eluent concentration/pH/flow rate gradient elution. Column Chromolith RP coated with DDMAU 200 x 4 6 mm Eluent A: 5 mM acetic acid, pH 3.6. Eluent B· 100 mM ammonium acetate, pH 5.3.

Gradient: 0 0 - 8.0 min – 100 % A, F = 0.5 mL/min,

8.0 - 14.0 min –100 % A to 50 % A – 50 % B, F = 0 5 to F = 2.0 mL/min,

14.0 - 22.0 min – 50 % A – 50 % B to 100 % B, F = 2.0 mL/min;

22.0 - 28.0 min – 100 % B, F = 2 0 to 3.5 mL/min;

28.0 - 32.0 min – 100 % B, F = 3.5 to 4.5 mL/min;

32.0 - 50.0 min – 100 % B; F = 4.5 mL/min.

Column was thermostated at 8.5 °C. Direct UV detection at 260 nm

The samples were extracted using the perchloric acid method detailed in Section 2.4.6. Acumedia, Bacto, Difco and Invitrogen yeastolates and yeast extract itself were analysed and chromatograms obtained are overlaid to show the differences between samples. As can be seen from Fig. 6-3-2, a series of unidentified peaks were present on the chromatograms, although a number of the above precursors were also identified using standard addition.

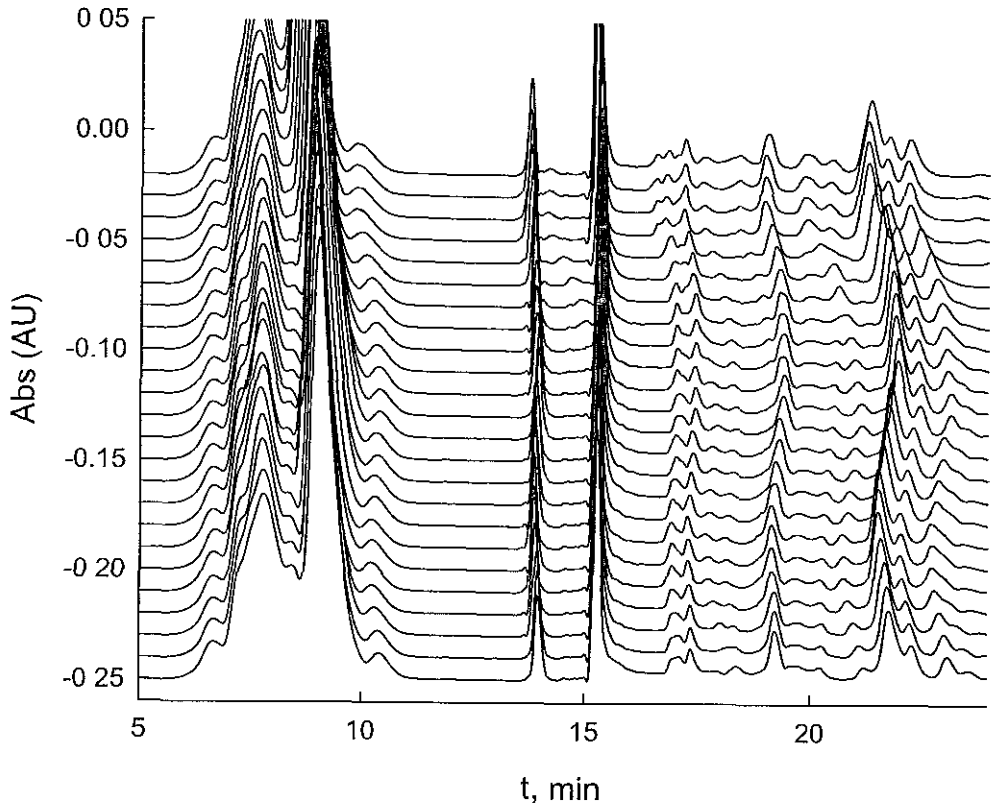


Figure 6-3-3. Overlaid chromatograms of commercial yeastolate extract samples using triple eluent concentration/pH/flow rate gradient elution. Column Chromolith RP coated with DDMAU 200 x 4 6 mm Eluent A: 5 mM acetic acid, pH 3.6. Eluent B: 100 mM ammonium acetate, pH 5.3

Gradient: 0.0 - 8.0 min – 100 % A, F = 0.5 mL/min;

8.0 - 14 0 min – 100 % A to 50 % A – 50 % B, F = 0.5 to F = 2.0 mL/min;

14 0 - 22.0 min – 50 % A – 50 % B to 100 % B, F = 2.0 mL/min;

22 0 - 28.0 min – 100 % B, F = 2.0 to 3.5 mL/min;

28.0 - 32.0 min – 100 % B, F = 3.5 to 4 5 mL/min;

32.0 - 50 0 min – 100 % B; F = 4.5 mL/min.

Column was thermostated at 8.5 °C. Direct UV detection at 260 nm

At the same time the stability of samples extracted using perchloric acid method was studied. For this Bacto yeastolate sample was injected every 90 min. This

procedure was repeated 18 times in the same conditions as described above. The resultant overlaid chromatograms are shown on Figure 6-3-3. It can be seen that yeastolate sample degrades quite rapidly over time. This might occur due to bacterial growth, or the absence of preserving agents.

6.4. Separation of nucleotides on ultra-short columns

6 4.1. Separation of nucleotides on monolithic ultra-short column

The use of ultra-short monolithic columns or "discs" (from 4 to 50 mm in length) have attracted attention due to their potential for rapid separations of relatively simple samples, with applications in several areas, including biochemistry, pharmaceutical analysis and affinity chromatography [29-31]. At the same time, low column backpressures with such ultra-short columns allow the application of high flow rates (up to 5 mL/min) in order to further reduce retention times. Such elevated flow rates can be used at a constant set rate (isofluentic mode), or with an applied flow programme, as it was discussed in Chapter 3. Here a DDMAU modified monolithic micro-column (10 x 4.6 mm) was used for the separation of nucleotides. The chromatogram obtained using a flow gradient was compared with one obtained under isofluentic conditions. Flow rates between 0.2 and 3.0 mL/min were investigated using the column. At flow rate of 3.0 mL/min the backpressure was < 70 atm. Under isofluentic conditions of 0.2 mL/min, a mixture of nine nucleotides was separated with the DDMAU modified micro-column in an excessive 400 min. This chromatogram is shown as Figure 6-4-1. Increasing the column flow rate to a constant rate would obviously reduce total run time, but also resulted in a considerable reduction in resolution of the weakly retained closely eluting analytes. For strongly retained species this was not significant. The same nucleotide mixture was run with a flow gradient applied from 0.2 to 3.0 mL/min for 30 min and then maintained at 3.0 mL/min for next 20 min. The chromatogram obtained under these conditions is shown on Fig. 6-4-2. Resolution of weakly retained nucleotides was maintained, but the overall run time was reduced to 35 min. Although in this case, while this cannot be considered rapid, it does show the potential of short monolithic phases for simple separations, and here represents approximately 90 % reduction in retention time without significant loss of resolution.

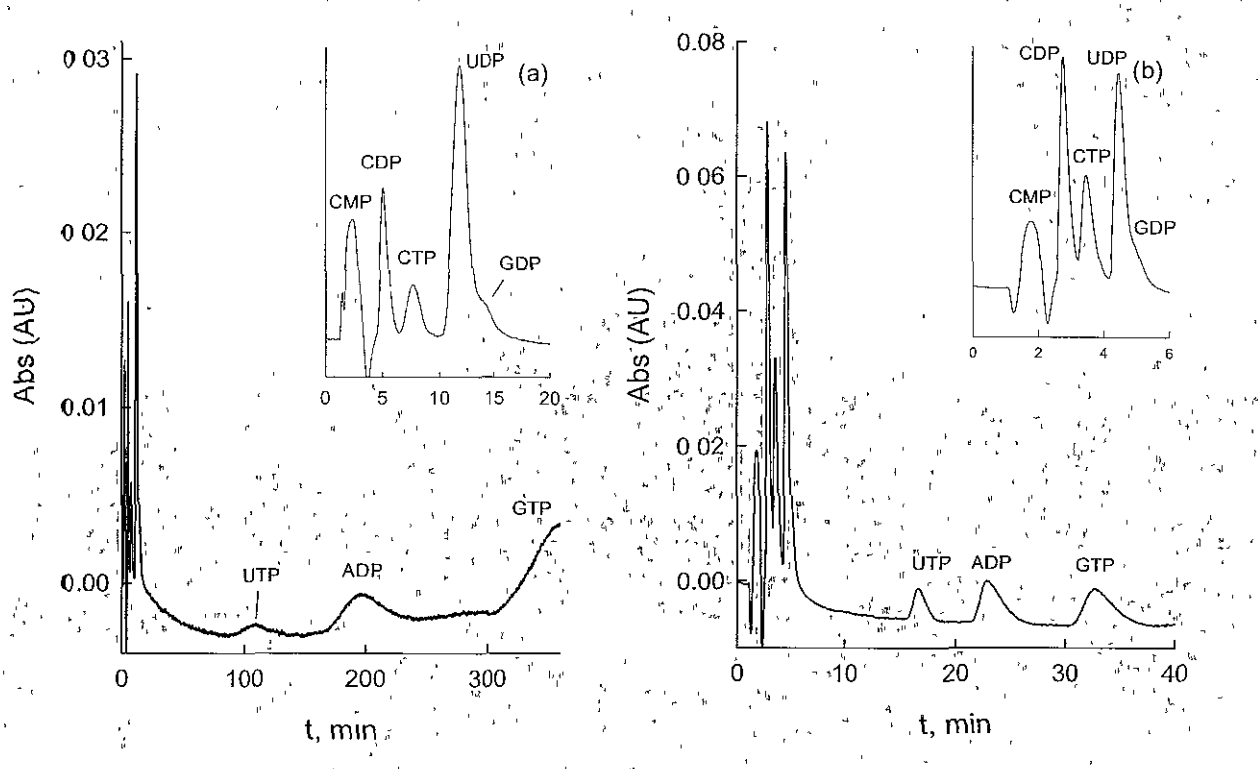


Figure 6-4-1. Separation of nucleotides on DDMAU monolithic micro-column. Column. Chromolith RP SecurityGuard cartridge 10 x 4 6 mm I D., coated with DDMAU. Eluent: 5 mM ammonium acetate buffer, pH 4 8. Direct UV detection at 260 nm

(a) Isofluentic conditions, F = 0.2 mL/min

(b) Flow gradient applied:
0.0 min to 30 0 min – F = 0.2 mL/min to 3.0 mL/min;
30.0 min to 50 0 min – F = 3.0 mL/min

6.4.2. Separation of nucleotides on particle packed RP ultra-short column

A DDMAU modified packed Gemini C_{18} micro-column was used for separation of nucleotides. The chromatogram obtained in isofluentic mode, at a flow rate of 1.0 mL/min resulted in a column backpressure of only < 30 atm. Under these conditions a mixture of 6 nucleotides was separated in approximately 30 min. This chromatogram is shown as Figure 6-4-2. Again while this separation cannot be considered rapid, it does show the potential of ultra-short columns for simple separations.

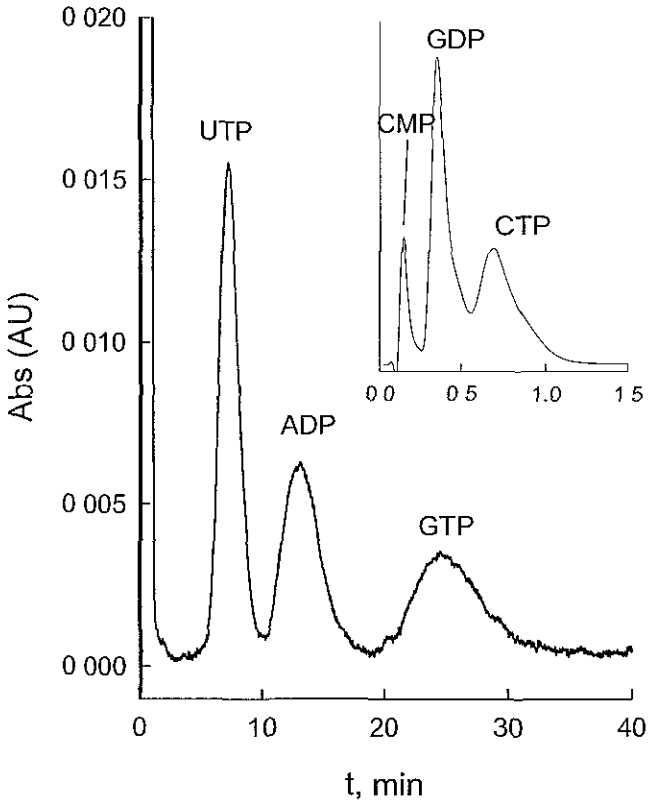


Figure 6-4-2. Separation of nucleotides on DDMAU modified packed micro-column. Column: SecurityGuard Gemini C_{18} cartridge 4.0 x 3.0 mm I.D., coated with DDMAU. Eluent: 5 mM ammonium acetate buffer, pH 4 8. F = 1.0 mL/min. Direct UV detection at 260 nm

6.5. Conclusions

The results presented in this Chapter illustrate the possibility of the simultaneous separation of analytes, present within a sample in cationic, neutral and anionic forms on a zwitterionic monolithic stationary phase. The method utilised the unique characteristics of the DDMAU coated monolith for the development of a triple gradient approach, which will no doubt gain in popularity for future users of monolithic columns for complex and diverse mixtures of analytes.

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CONCLUSIONS

An investigation into novel zwitterionic ion-exchangers prepared by the dynamic coating of particle packed (Gemini C_{18}) and monolithic (Chromolith) reversed-phase columns with the carboxybetaine type surfactants DDMAU and DDMAB, has been carried out. The adsorption of surfactants was investigated and possible structures of adsorbed layers were proposed. It was found that coating type and column capacity were dependent on pH and ionic strength as these factors had the strongest affects upon surfactant micelle and internal salt formation. The effect of the eluent cation charge on anion retention was also studied It was shown that the model for the separation mechanism developed for sulphobetaine type surfactants was also applicable to carboxybetaine type phases. A new cation charge gradient elution concept was proposed and its application demonstrated for the reduction of separation time by almost 50 % for a mixture of test anions.

The effect of eluent concentration and pH on separation of anions on particle packed and monolithic RP columns coated with either DDMAU or DDMAB was studied. The separation conditions for each column were optimised using product resolution criterion and resultant response surface. An attempt to apply a pH gradient to the separation was made and investigation of column capacities has been carried out.

The investigation of a short DDMAU micro-column for ion chromatographic separations has been carried out. A flow gradient approach was applied to the micro-column in order to reduce excessive run times, which was possible due to the relatively low backpressures generated over a column of such short dimensions. The micro-column exhibited stable retention times and linearity over a wide range of analyte and matrix anion concentrations, and a low affinity to chloride allowed the column to be applied directly to saline samples.

The effect of eluent nature and pH on the separation of cations on particle packed and monolithic RP columns coated with DDMAU was studied. The separation conditions on each column were optimised. An investigation into the mechanism of cation retention on zwitterionic stationary phases was made. It was shown that with citrate eluent separation occurs according to an anion-exchange mechanism, as alkaline-earth and transition metals form anionic complexes. Using oxalate eluent, separation occurs according to a cation-exchange mechanism for alkaline-earth metals, as they form neutral 1:1 neutral complexes and according to an anion-exchange mechanism for transition metals. Compared to the pervious studies, which utilised polyaminocarboxylic

complexones for the simultaneous separation of anions and cations, the improved selectivity was achieved with the DDMAU coated columns and citrate eluent system resulting in faster separation of more species in shorter time with no sample pre-treatment needed.

Finally, the developed DDMAU zwitterionic ion-exchangers were applied to the separation of nucleotides, nucleosides and their bases, which were present in anionic, cationic and neutral forms. The method utilised the unique characteristics of the DDMAU coated monolith for the development of a novel triple concentration/pH/flow rate gradient approach.