Influence of capacity on the retention characteristics in Zwitter Ion Chromatography (ZIC) and ZIC-Hydrophilic Interaction Chromatography (HILIC) on four different sulfobetaine stationary phases

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M.Sc. Ashraf Saad Rasheed

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Erstgutachter: Prof. Dr. Andreas Seubert
Zweitgutachter: Prof. Dr. Ulrich Tallarek

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Zusammenfassung

Ashraf Saad Rasheed

Einfluß der Kapazität auf die Retentionseigenschaften in der Zwitterionenchromatographie (ZIC) und in der ZIC-Hydrophile Interaktions Chromatographie (HILIC) für vier verschiedene Sulfobetain-Austauscher

Schlüsselwörter: Zwitterionenchromatographie, Hydrophile Interaktionschromatographie, Sulfobetaine, Kapazität, Pharmazeutika, Überschussadsorptionsisothermen.

Der Einfluss der Kapazität auf die Trenneigenschaften einer Reihe von zwitterionischen Sulfobetain-Austauschern wurde untersucht. Die Materialien wurden durch radikalische Pfropfpolymerisation von PS/DVB-Polymeren hergestellt. Neben der Kapazität haben sich die zwitterionischen Austauscher in ihrem Abstand zwischen den Ladungen unterschieden, wobei zwischen einer (SB1) und bis zu vier Methylengruppen (SB2 bis SB4) variiert wurde. Der wichtigste Aspekt dieser Arbeit ist die Untersuchung des Einflusses einer veränderten Kapazität der zwitterionischen stationären Phase auf deren Verhalten im ZIC und im ZIC-HILIC-Modus. Die zwitterionischen stationären Phasen zeigen bezüglich der Kapazität insgesamt ein sehr ähnliches Verhalten unter ZIC und ZIC-HILIC Bedingungen. Eine Erhöhung der Kapazität der Sulfobetain-Austauscher führt zu verschiedenen Wechselwirkungen anionischer Analyten mit den kationischen Gruppen der Sulfobetain-Austauscher. Diese Interaktionen werden durch die unterschiedliche Flexibilität der Sulfobetain-Ketten bestimmt, da ihre Fähigkeit zur Ausbildung intra-und intermolekularer Ionenpaare variiert. Der Abstand der Ladungen und die Austauschkapazität der Materialien hat somit einen Einfluß auf die Retention der anionischen Analyten.

Die Kapazität der Sulfobetain-Austauscher spielt eine bedeutende Rolle bei der Trennung von Anionen. Im ZIC Modus zeigt der SB2-Austauscher eine anderes Verhalten im Vergleich zu einer früheren Arbeit [10]. Ursache hierfür ist die niedrigere Kapazität des in der früheren Arbeit eingesetzten Materials (130 ueq g^{-1}). Diese Vorgängerstudie zeigte eine konstante Retention für Anionen bei Varation der Eluentionenkonzentration. In dieser Arbeit konnte unter Verwendung höherkapazitiver SB2-Austauscher (300-790 ueq g^{-1}) gezeigt werden, dass die Retention für Anionen mit zunehmender Eluentionenkonzentrationen abnimmt. Der ist die Trennmechanismus von Anionen auf diesen SB2-Austauscher folgt somit einem reinen Anionenaustauschmechanismus. Insgesamt zeigte die Untersuchung des Einflusses der Kapazität auf das Elutionsverhalten bei der Trennung von anorganischen Anionen keine signifikanten Änderung der Trennselektivität und Effizienz.

Der Retention von Analyten im ZIC-HILIC-Modus fehlt immer noch eine widerspruchsfreie theoretische Erklärung. Die Untersuchung einer Reihe von Sulfobetain-Austauschern soll mehr Einblick in den Mechanismus der Trennung in diesem Modus geben. Zwitterionsiche Austaucher des Typ's SB1, SB3 und SB4 verhalten sich wie bislang für ZIC-HILC Materialien erwartet. Dagegen wird bei Materialien auf SB2-Basis ein RP-ähnliches Verhalten gefunden. Das unterschiedliche Verhalten von SBn-Säulen kann nur auf die unterschiedliche geometrische Ausrichtung der Sulfobetain-Moleküle zurückführen sein. Interessanterweise zeigt der SB3-Austauscher einige Selektivitätsänderungen bei der Elution von Anionen, wenn der Einfluß des Acetonitril-Gehalts untersucht wird. Diese Änderung ist auf die Überlagerung von chaotropen und hydrophilen Wechselwirkungen und der Hydratationsenthalpie von Anionen zurückzuführen.

Bei SB4-Materialien wird unter ZIC-HILIC Bedingungen bei niedrigen Eluentionenstärken eine Veränderung der Selektivität durch Beeinflussung der quasi-immobilisierten Wasserschicht auf dieser Art von zwitterionischen stationären Phasen beobachtet. Dieses Retentionsverhalten von Anionen kann nur durch elektrostatische Wechselwirkungen verursacht werden. Am Ende wird der Trennmechanismus wahrscheinlich eine Überlagerung von hydrophilen und elektrostatische Wechselwirkungen am Sulfobetain stationären Phase sein. Ansonsten wurden keine Änderungen in der Selektivität von Anionen gegenüber dem ZIC-Modus beobachtet.

Die aktuelle Arbeit untersucht die Retentionseigenschaften von acht pharmazeutischen Verbindungen auf den vier Sulfobetain-basierten stationären Phasen unter HILIC-Bedingungen unter Verwendung eines bislang einzigartigen Säulenvergleichstests mit reinen Anion- bzw. Kationenaustauscher. Diese Vergleichssäulen basieren auf dem gleichen Grundmaterial, verwenden die gleiche Funktionalisierungsstrategie und weisen ähnliche Kapazitäten auf. Die Retention der Pharmazeutika basiert einem gemischten Modus basierend auf RP, ZIC-HILIC und Ionenaustausch-Wechselwirkungen in unterschiedliche Ausprägung. Die Untersch-eidung zwischen RP- und ZIC-HILIC-Modus erfolgte anhand der Variation des Acetonitril-Anteils, die auf Ionenaustausch basierende Retention wurde anhand von Variationen der Eluentionenkonzentration und des Säulenmaterials ermittelt. Der Grund für dieses komplexe Verhalten ist der unterschiedliche strukturelle Aufbau der Pharmazeutika.

Bei Auftragung des logarithmischen Retentionsfaktors gegen die logarithmische Acetonitril-Konzentration in der mobilen Phase wird eine Korrelation $R^2 > 0{,}94$ aufgefunden. Die geometrischen Ausrichtung der zwitterionischen Materialien spielt eine bedeutende Rolle in der Beziehung zwischen $\log k'_w$ und $\log P$ und zeigt eine ausgezeichnete Übereinstimmung zwischen den experimentellen und den berechneten Werten.

Diese Reihe von stationären Phasen sind ein neues Werkzeug, das verwendet werden kann, um die Retentionsmechanismen von multifunktionellen Verbindungen wie z.B. Pharmazeutika zu untersuchen. Außerdem kann es helfen, die geeignete stationäre Phase für komplexe analytische Probleme in der pharmazeutischen, biologischen und Umweltproben auszuwählen.

Die zwitterionische Ionenchromatographie eignet sich in Online-Kopplung an einen elementspezifischen Detektor wie ICP-AES für die Untersuchung der Komplexbildungseigenschaften von Deferoxamin (DFOM). DFOM ist ein starker Chelatligand für mehrfach geladene Ionen wie Fe(III) und Al(III), die vom biologischen Standpunkt aus gesehen wichtige Ionen darstellen. Es konnte gezeigt werden, daß Metall-DFOM-Komplexe auf Sulfobetain-Materialien hauptsächlich durch Kationenaustausch getrennt werden. Trotz zahlreicher Veröffentlichungen in der Literatur, die besagen, dass zwitterionische Materialien in der Regel für die Trennung von Anionen gut geeignet sind, hatte bislang keine Studie kationische organische

Verbindungen untersucht. Die aktuelle Studie bestätigt die Fähigkeit der ZIC-HILIC Säulen, Metall-Komplexe, organische Kationen und unpolare Verbindungen zu trennen. Trotzdem sind weitere Experimente nötig, um das mechanistische Wissen um die Trennungen im ZIC-HILIC-Modus für solche Verbindungen zu erweitern.

Die Variation der Zusammensetzung von Acetonitril/Wasser-Gemischen führt auf Sulfobetain-Austauschern durch die Anwesenheit von polaren sowie unpolaren funktionellen Gruppen auf ihrer Oberfläche zu Veränderungen in der Wasser-bzw. Acetonitril-Anreicherung an der Grenzschicht. Es wurde festgestellt, dass die Sulfobetain-Austauscher Wasser anreichern, wenn der Wasseranteil im Eluent gering ist. Interessanterweise spielt die Spacerlänge zwischen den geladenen funktionellen Gruppen und auch die Kapazität der zwitterionischen Materialien eine wichtige Rolle führt die Ausbildung dieser Wasserschicht an der Grenzfläche.

Abstract

Ashraf Saad Rasheed

Influence of capacity on the retention characteristics in Zwitter Ion Chromatography (ZIC) and ZIC-Hydrophilic Interaction Chromatography (HILIC) on four different sulfobetaine stationary phases

Keywords: Zwitter ion chromatography, Hydrophilic ion chromatography, Sulfobetaine, Capacity, Pharmaceutical, Excess adsorption isotherm.

A number of covalently bonded zwitterionic stationary phases with inner quaternary amines and outer sulfonic acids in varying capacities was synthesized based on poly (styrene-DVB) particles by graft polymerization. The different spacer lengths and capacities are used as an investigative tool, for the retention behavior of the inorganic anions in ZIC and ZIC-HILIC modes. The separation mechanism is explored by varying eluent ionic strength, eluent pH and the volume fraction of organic modifier. Remarkably, the increasing capacity exhibits different characteristics of anions retention for all exchangers in two modes. Hence, this leads to increased, decreased or no significant change in the retention times of inorganic anions. Moreover, no change in the separation mechanism and selectivity of anions occurred when investigating the influence of eluent concentration, pH of eluent and acetonitrile content for various capacities of zwitterionic columns. It should be noted that high values of the capacity of the SB2-exchanger exhibit a different separation mechanism for inorganic anions in comparison to a previous study made for the ZIC-mode. Furthermore, a new correlation between spacer length and the capacity and their impact on the retention time of anions in ZIC and ZIC-HILIC modes were found. The results of variations of acetonitrile content, buffer concentration and mobile phase pH show that pharmaceuticals can be sepa-

rated based on hydrophilic, hydrophobic and anion/cation-exchange interactions between the stationary phase and analyte. For a differentiation between the separation mechanism for the first time a set of cation and anion exchangers based on the same core material has been used as investigative tool. In addition, we have proven the suitability of ZIC-HILIC stationary phases for the determination of the chromatographic hydrophobicity parameter.

The pharmaceutical deferoxamine mesylate (DFOM) is a chelating agent which forms complexes with multiple charged metal ions of biological importance (Fe^{3+}, Al^{3+}) and other metals. We could demonstrate that the sulfobetaine exchangers are able to separate the Fe(III)-DFOM and Al(III)-DFOM complexes by IC-ICP-AES. The results of the eluent ionic strength and pH variations show that metal-DFOM complexes were separated based on a cation exchange mechanism.

Excess adsorption isotherms of water from acetonitrile were measured for ten zwitterionic stationary phases (eight sulfobetaine exchangers have been prepared and two commercially available stationary phases) using the minor disturbance method. The retention factors of the eight pharmaceutical compounds (Deferoxamine mesylate, Thiamine.HCl, Diclofenac sodium, Cyclopentolate.HCl, Dexamethasone sodium phosphate, Tetracaine.HCl, Pilocarpine .HCl and Chloramphenicol) can be correlated with the maximum excess of water adsorption.

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List of abbreviations

ACN	Acetonitrile
B1	Thiamine hydrochloride
CAP	Chloramphenicol
CPH	Cyclopentolate hydrochloride
DFOM	Deferoxamine mesylate
DMSP	Dexamethasone sodium phosphate
DCS	Diclofenac sodium
DMAES	2-(Dimethylamino) ethanesulfonic acid
EDL	Binary electrical double layer
ESI-MS	Electrospray ionization mass spectrometry
GC	Gas chromatography
GLC	Gas liquid chromatography
GSC	Gas solid chromatography
HDPE	High-density polyethylen
HILIC	Hydrophilic interaction chromatography
HEMA-EDMA	2-Hydroxyethyl methacrylate-ethylene dimethacrylate
IC	Ion chromatography
IES	Ion exchange chromatography
ICE	Ion exclusion chromatography
IPC	Ion pair chromatography
ICP-AES	Inductively coupled plasma atomic emission spectrometry
IP	Isoelectric point
LLC	Liquid liquid chromatography

LSC	Liquid solid chromatography
MDM	Minor disturbance method
NP-LC	Normal phase liquid chromatography
PCH	Pilocarpine hydrochloride
PEEK	Polyether ether ketone
PS/DVB	Polystyrene/divinylbenzene
RP-HPLC	Reversed phase-high performance liquid chromatography
RP-LC	Reversed phase liquid chromatography
SB	Sulfobetaine
SB1	Sulfobetaine exchangers with one-methylene groups between the charged groups
SB2	Sulfobetaine exchangers with two-methylene groups between the charged groups
SB3	Sulfobetaine exchangers with three-methylene groups between the charged groups
SB4	Sulfobetaine exchangers with four-methylene groups between the charged groups
TCH	Tetracaine hydrochloride
TLC	Thin layer chromatography
ZIC	Zwitterionic ion chromatography

1 Introduction and objectives

Zwitterionic ion chromatography (ZIC) is a new trend of ion chromatography (IC), which uses zwitterionic stationary phases for anion and cation separations. The concept combines both anionic and cationic functional groups in a single molecule on the stationary phase in order to improve or render the ion exchange selectivity [1, 2]. This new form of ion chromatography was initially proposed in 1993 by the *Hu et al.* [3], using a strong/strong charged zwitterionic micellar coated stationary phase. There are a number of benefits of ZIC when compared to conventional IC. Firstly, separations of inorganic salts by ZIC can be accomplished using water [4] or very dilute electrolyte solutions as mobile phase [5-7], in order to provide optimal conditions for conductivity detection of inorganic ions [4] and, therefore, increases in sensitivity of conductivity detection. Secondly, ZIC overcomes several difficulties in the analysis of high matrix samples such as seawater samples [8]. Thirdly, ZIC overcomes the major constraints (e.g. pretreatment of samples, overlapping peaks of NO_2^- and Cl^- and a change in separation efficiency, column-pressure and retention time) encountered in the IC analysis of inorganic anions in urine and serum by adding zwitterionic micelles to the mobile phase [9].

Sonnenschein et al. [10] investigated the polarities of zwitterionic monomers by RP-HPLC and gave the explanation that polarity should be dependent on the ability of the monomers to compensate their charges via intramolecular interaction. In their work, they found that the SB2 material with two-methylene spacers between the charges shows the highest polarity compared to SB1, SB3 and SB4 exchangers. The reason for this must be the chain length between charged groups. With increasing spacer length, the sulfobetaine molecules on the PS/DVB surface remain flexible with increasing ability to the formation of both intermolecular and intramolecular excluding the SB1 material. *Nesterenko et al.* [11] investigated the effect of the distance length between positively-negatively charged using C_{18}-materials dy-

namically coated with carboxybetaine type surfactants with three to ten methylene groups between the anion and cation sites.

In their study, no methylene groups related changes in the behavior were observed. The mechanism of ZIC has not been fully investigated and it should be possible to extend the mechanistic knowledge about ZIC using the capacity as diagnostic tool. There is a number of separation mechanisms engaged in ZIC. A binary electrical double layer model suggested by *Hu et al.* [8, 12] postulates an enrichment of cations at the negatively charged and of anions on the positively charged functional groups of the zwitterionic surface. The cation cloud around the anionic functional group should allow the retention of anions. Additional effects on the separation mechanism of the inorganic ions by the EDL can be explained by the idea of ion-pair formation [3]. *Cook et al.* [13, 14] have been suggested a Donnan membrane mechanism based on both a shield effect and the chaotropic interaction.

Despite the availability of numerous studies in ZIC mode, no investigation has been carried out for the effect of the capacity of ZIC materials. **The first objective** of our work is to investigate the influence of the capacity of the zwitterionic stationary phases on the separation selectivity and efficiency for anions using ZIC mode. To study this, a variation of the amount of sulfobetaine monomers used for grafting polymerizations permitted the preparation of four sulfobetaine exchangers per charge distance with different capacities**.**

Hydrophilic interaction liquid chromatography (HILIC) performed on zwitterionic stationary phases (ZIC-HILIC) is an upcoming separation technique with rapidly increasing importance. It is suitable for the separation of hydrophilic and ionic substances. These ZIC-HILIC materials can be operated in different separation modes depending on the mobile phase composition. The commonly used HILIC mode requires high concentration of organic solvents and allows separation with normal phase chromatography behavior. HILIC is therefore an alternative to normal phase liquid chromatography (NP-LC). However, the mechanism used in HILIC is more complicated than that in NP-LC. The mobile phase, used in HILIC, is comparable to this used in the reversed phase liquid chromatography (RP-LC) but it contains usually more organic mobile phase [15]. In 1975, the first generation of HILIC mode separations begun by *Linden et al.* [16] who separated saccharides using an amino-silica phase. In 1990, *Alpert* first coined the term HILIC [17]. In his work, he used hydrophilic stationary phases

(poly sulfoethyl aspartamide and poly hydroxyethyl aspartamide) for separating peptides, nucleic acids and carbohydrates. The stationary phases in HILIC are classified into three groups based on the charge properties of the functional groups: neutral, charged and zwitterionic phases [18]. Notwithstanding the numerous studies in the ZIC-HILIC mode, the influence of capacity and the spacer length between the charges has not been investigated before.

The second objective of our work is the investigation of the impact of the capacity of the zwitterionic stationary phases in the ZIC-HILIC mode. Will there be a correlation between the spacer length and the separation selectivity and efficiency for inorganic anions? Another interesting question is, whether it is possible to extend the mechanistic knowledge about ZIC-HILIC using the capacity information and the difference in the length of chain between the two charges. *Alpert* [17] proposed that the separation mechanism for HILIC was occurring between two layers, the water-enriched layer on a stationary phase and a mainly organic mobile phase; the analyte is distributed between the water-enriched stationary layer and the mobile phase with low water contents**.**

The upcoming questions are now “What is the influence of varying eluent ionic strength, eluent pH and the volume fraction of organic modifier on selectivity order and separation mechanism of inorganic anions in ZIC and ZIC-HILIC modes?” The further interesting question is “Is there a correlation between the chain length and capacity and its impact on the retention characteristics of anions and other analytes in ZIC and ZIC-HILIC modes?”

The polar functional groups of ZIC-HILIC stationary phases should have an influence on water adsorption in hydrophilic interaction chromatography mode and therefore they should provide more information about the most appropriate columns for HILIC-applications. However, for more than 30 years, the majority of the studies have concentrated on the adsorption isotherm on reversed stationary phases [19-22]. Consequently, **the third objective** of the work is the investigation of excess adsorption isotherms for water on our sulfobetaine stationary phases and the comparison with other commercial ZIC-HILIC columns. The variable SBn-spacer and capacity should have an influence on the water enrichment at column surface and may be used for the development of phases with higher water enrichment at the surface. We believe that this has not been investigated previously in the literature.

HILIC is a fast growing alternative to traditional HPLC for the analysis of pharmaceuticals. The poor retention of polar pharmaceuticals on most reversed phase materials (RP) remains a big challenge and HILIC offers an attractive alternative and can be used as an orthogonal separation method to RP-HPLC [23]. At present, HILIC is attracting much attention because it solves many difficult separation problems. It has been successfully applied to the analysis of saccharides [16], sugar [24], cocaine in serum [25], pharmaceutical compounds [26-28], metabolites [29], toxins [30], carbohydrates [31-33] and peptides [34-37]. Promoting the understanding of retention behavior in HILIC will increase the range of possible applications.

Remarkably, HILIC materials have hydrophilic, hydrophobic and ionic groups and they should be able to activate almost every type of interaction with analytes. Pharmaceutical compounds are often molecules with hydrophilic, hydrophobic and ionic groups and therefore ideally suited to study the retention characteristics of HILIC materials. Our column test set with different HILIC columns is ideally suited to investigate the influence of the spacer length on the separation characteristics and has not been investigated before. Although the use of HILIC has been growing widely over the past decade, parts of its mechanism still remain unresolved until today.

The fourth objective of my work is therefore the investigation of the separation characteristics of polar and non-polar pharmaceutical compounds on sulfobetaine stationary phases with different numbers of methylene spacers in HILIC mode. Some of the pharmaceutical compounds can act as a strong chelator for multiple charged metal ions of biological importance. It will be interesting to see if the metal–complexes are also separately using the SBn-columns in an IC-system, which is on-line coupled to ICP-AES as an element specific detection.

2 Principles of ion chromatography

2.1 Introduction to chromatography

Chromatography is a variety of physicochemical techniques for the separation of components within mixtures; all methods are based on the distribution of a component between two immiscible phases, the stationary phase and mobile phase. The chromatographic methods are divided according to the physical state of these phases [38]. The Russian botanist, *Mikhail Tswett* [39, 40] was the first who invented chromatography based on a column technique to separate leaf pigments on a polar solid phase.

2.1.1 Basic concepts

The principle of chromatography is based on the repetitive distribution of a solute between two immiscible phases. The substance to be analyzed is dissolved in the mobile phase and passes through a stationary phase, whereas the mobile phase may be liquid, gaseous or a supercritical fluid. Solutes are injected at the beginning of the chromatographic column. The solutes are distributed between the stationary phase and the mobile phase, according to the distribution coefficient. The phases are chosen so that the analytes are distributed between the mobile and stationary phases to different degrees. The solute with the lower affinity to the stationary phase travels via the column more quickly. In contrast, analytes those are strongly retained by the stationary phase travel tardily, and, therefore, the analytes leave the column at different times (so-called retention times) and can be detected externally [41]. These differences in the travel rates separate the analytes into discrete bands, which can be analyzed quantitatively and qualitatively. The common methods used are gas chromatography (GC) and high performance liquid chromatography (HPLC). There are varieties of ways to classify the various chromatographic techniques.

The classification is based on the physicochemical interaction of the solute with the mobile and stationary phases. The first classification of chromatographic techniques is according to the nature of the mobile phase. These classifications are summarized in Table 2.1, so the primary distinction in Table 2.1 is between gas chromatography GC (gaseous mobile phase) and liquid chromatography (liquid mobile phase). Another difference in the GC is based on the nature of the stationary phase, solid or liquid, as in the cases gas-solid-chromatography (GSC, adsorption) and gas-liquid-chromatography (GLC, partition).

Categorizing LC techniques are more complicated. LC techniques are subdivided into two general categories: planar and column chromatography. The first category is planar chromatography. The classification depends on the stationary phase; it can be a liquid adsorbed (paper chromatography, PC) or solid particles (thin layer chromatography, TLC). The second category (column chromatography) has been classified according to both, the phase and the mechanism of the phase distribution, as in the cases liquid-solid-chromatography (LSC) and liquid-liquid-chromatography (LLC) [41, 42].

Table 2.1: Classifications of chromatographic methods

	Gas mobile phase	Liquid mobile phase
Solid stationary phase	GSC (gas-solid-chrom.)	LSC (liquid-solid-chrom.)
Liquid stationary phase	GLC (gas-liquid-chrom.)	LLC (liquid-liquid chrom.)

2.1.2 Retention parameters

In a chromatographic method, we observe a dynamic equilibrium for analytes between the phases involved. This balance is the equilibrium distribution. A successful separation is given only if the distribution coefficient D_A of the substances to be separated is sufficiently differ-

ent. D_A is defined as the ratio of the concentration of a substance (A) between mobile ($_M$) and stationary phase ($_S$). Substances with a high distribution coefficient D_A are more strongly retained by the stationary phase than those with small distribution coefficients [43].

$$D_A = \frac{C_{AS}}{C_{AM}} \tag{2.1}$$

In a chromatographic column, two different analytes are separated if they spend different times in the stationary phase as in Figure 2.1 (a). The time necessary for the non-retained analytes to move is called the hold-up time t_M, also sometimes referred to as dead time or void time. The analyte retention time t_S is defined as the time for solutes not to move along the column [38]. As in Equation 2.2, the gross retention time or residence time t_R of analytes on the stationary phase is obtained from the analyte retention time and column hold-up time:

$$t_R = t_S + t_M \tag{2.2}$$

When injection is made at time t = zero. The non-retained signal shows at $t_R = t_M$ and is called the hold-up time; two retained signals (analyte affinity for the stationary phase) show at time t_{R1} and t_{R2} (retention times of signals A and B respectively). This is illustrated in Figure 2.1 (a) (idealized chromatogram).

The retention volume V_R is calculated from the solute retention time and a constant of flow rate of mobile phase F:

$$V_R = t_R F \tag{2.3}$$

The asymmetry factor A_S is defined as the ratio of the distances (tail portion-b and front portion-a) between the central verticals and the slopes of the distribution at 10% of their height as shown in Figure 2.1 (b).

As in Equation 2.4, the asymmetry factor A_S is calculated of peak distortion:

$$A_S = \frac{b}{a} \tag{2.4}$$

Both *a* and *b* are measured at 10% of the peak height as shown in Figure 2.1 (b). The individual analytes behave independently of one another through the chromatographic process.

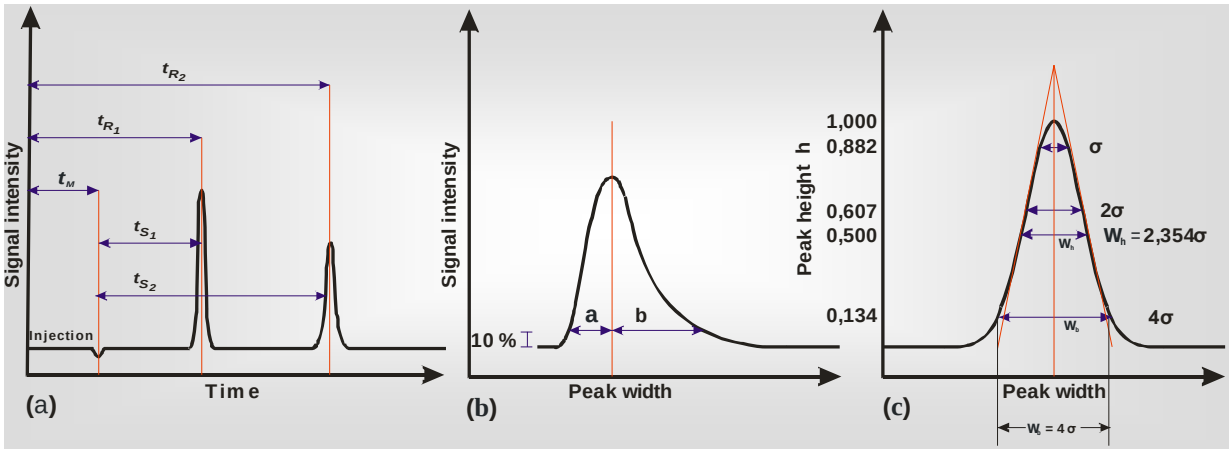


Figure 2.1: (a) Idealized chromatogram. (b) Definition of the asymmetry factor. (c) Gaussian distribution with characteristic parameters [38].

Consequently, after repeated sorption and desorption of solutes on the stationary phase, they produce a randomized aggregation of retention times. Sometimes some unwanted interaction occurs through the chromatographic process. This is usually indicated by non-symmetrical signals. In the actual sample, asymmetric signals can be categorized as tailing or fronting depending on the style of the asymmetry. For asymmetry factors > 1, the asymmetry is called tailing. Tailing effects occur via a fast increase of the chromatographic signal followed by a relatively slow decrease; primarily responsible for this effect are adsorption processes.

For asymmetry factors < 1, the asymmetry is called fronting. The image of the peak shape of this effect is the opposite of the tailing, fronting effect which happens if the stationary phase does not have a sufficient number of suitable adsorption sites [38].

2.1.3 Retention factor, selectivity and resolution

The retention factor *k'* indicates the factor by which the analyte is staying longer on the stationary than in the mobile phase. Mathematically, it is defined as a product of the distribution coefficient D_A and the ratio of the volume of the stationary phase V_s to the mobile phase as shown in Equation 2.5:

$$k' = D_A . \frac{V_s}{V_m} = \frac{C_s}{C_m} . \frac{V_s}{V_m} = \frac{t_{ms} - t_m}{t_m} = \frac{t_s}{t_m} \tag{2.5}$$

Small values of the retention factor *k'* mean that the analyte is eluted near the hold-up time; therefore, the separation will be poor. Large values of the retention factor k' mean that the longer the analysis time, the wider the peak and the lower the sensitivity [38]. For multicomponent systems, it is not only sufficient that the retention factor is in an acceptable range but also that they have to differ sufficiently from each other. To determine this parameter, the selectivity α is introduced, which refers to the relative retention of separation of two components. The selectivity is defined as the ratio of the analyte retention times of two different peaks as follows:

$$\alpha = \frac{t_{S_2}}{t_{S_1}} = \frac{t_{ms_2} - t_m}{t_{ms_1} - t_m} \tag{2.6}$$

If there are no thermodynamic differences between the two solutes under certain chromatographic circumstances, $\alpha = 1$ and coelution occurs, no separation is possible. The larger

value of α, means a better separation of analytes. With increasing selectivity, the time required for the separation of solutes also increases.

The ultimate goal of any chromatographic separation is to separate the solutes of a mixture into separate bands. A better measure to describe the quality of separation is the resolution *R*. The resolution *R* of two signals is defined as the difference between the distance of two peaks maxima divided by the arithmetic mean of peak width *w* at base:

$$R = \frac{t_{R_2} - t_{R_1}}{(w_1 + w_2)/2} = \frac{2\Delta t_R}{w_1 + w_2} \tag{2.7}$$

Where (t_{R1}, t_{R2}) are retention times for peaks 1 and 2, respectively, (w_1, w_2) are the widths of the peaks at baseline, as shown in Figure 2.1. If the difference in retention times of two peaks with the base width is large, we obtain a high resolution. A resolution of *R* = 2.0 (8σ-separation) is enough for quantitative analysis, but is not desirable because the items related to cost are too large for analysis. At a resolution of *R* = 0.5, it is still possible to be recognized as separate peaks of two analytes. A qualitative separation requires a resolution of R = 1; for quantification a resolutions in the range of R = 1.2-1.5 is required [38, 44, 45].

2.2 Theoretical concepts of the chromatography

The process to explain the mechanism of migration and separation of compounds on the column has been the source of considerable controversy. Therefore, there are two theories to explain the chromatography process. First, the plate theory (developed by *Martin and Synge* [46]) and second, the dynamic theory (proposed by *Van Deemter* [47]).

2.2.1 The plate theory

In 1940, *Martin and Synge* introduced the plate theory to describe chromatography by analogy to distillation and extraction [46]. It is sometimes beneficial to deal with the equilibrium

concept in chromatography; e.g. the theoretical plate value is a fanciful part of the column. The number of the theoretical plate *N* of a column can be calculated using the half-widths and the total retention times from the chromatogram:

$$N = \left(\frac{t_R}{\sigma}\right)^2 = 16\left(\frac{t_R}{w_b}\right)^2 = 5.54\left(\frac{t_R}{w_h}\right)^2 \quad (2.8)$$

Here, t_R is the retention time, σ is the peak standard deviation, w_b is the peak width at the baseline and w_h is the peak width at half height. The parameters σ, w_b *and* w_h can be obtained from the Figure 2.1 (c). The height equivalent of a theoretical plate *H* or *HETP* can also be used to describe the separation performance and is given by:

$$HETP = \frac{L}{N} = \frac{\sigma^2}{L} \quad (2.9)$$

Here, *L* is the column length. The concept of theoretical plates explains the appearance of Gaussian peaks. Namely, it is assumed that the compound passes down the column by transfer of the mobile phase from one plate to the other, due to irregularities in the equilibrium of the compound between the mobile and stationary phases caused by diffusion and continuous flow of the mobile phase, the compounds move through the column more slowly (interact strongly from plate to plate) or more rapidly (interact weakly from plate to plate). As a result, the narrow peaks with an increasing retention time of compounds on the stationary phase become broader.

We always observe some band broadening even for a non-retained signal. In some cases, it is advisable to calculate the effective plate number N_{eff} by using the corrected retention time t_R-t_m instead of the retention time t_R in Equation 2.8:

$$N_{eff} = \left(\frac{t_R - t_m}{\sigma}\right)^2 \quad (2.10)$$

The value of the number of theoretical plate *N* is usually used as an expression of the efficiency of a column. Smaller values of height equivalent of a theoretical plate *H* mean large values of *N*. Large values of *N* mean that the system is closer to equilibrium and therefore more efficient [42].

2.2.2 The dynamic theory (van Deemter equation)

In all of the above discussions in the theoretical plate model, the solute diffusion and the velocity of the mobile phase in the column were not taken into consideration. Consequently, the velocity must have an impact on the progress of the solutes in the column outlet. This dispersion affects the outcome of the quality of the analysis carried out [48]. There is no real equilibrium created between the analytes in the mobile and stationary phases, due to the always-flowing mobile phase in a chromatographic column. The peak broadening happens because of several effects occurring in the chromatographic column [42]. The first approach that deals with band broadening in chromatography was proposed by *Van Deemter* in 1956 [47]. The Van Deemter equation describes the factors affecting band broadening in a chromatographic separation. The van Deemter equation is:

$$H = A + \frac{B}{u} + Cu \tag{2.11}$$

Here *A*, *B*, and *C* are constant factors of multi-path effects, eddy diffusion, longitudinal diffusion, and mass transfer, respectively, and *u* is the average linear velocity of the mobile phase in the column.

Term A describes ***eddy diffusion***, also known as the packing factor. Non-retained analytes will not leave directly from the column inlet to the column outlet. The solute also faced particles of the stationary phase and it must move around them. Consequently, non-retained solutes may follow to a multi-pathway in their travel via the column [42].

The term A (eddy diffusion) in the van Deemter equation is:

$$A = 2\,\lambda\,d_P \tag{2.12}$$

In Equation 2.12, d_p is the average particle diameter and λ is an experimental packing factor (Coefficient describing the quality of the packing). The more homogenous particles size in the column (uniform particles), the closer the λ to one, therefore, it is an indication for the packing quality of the column [42].

Term B describes ***longitudinal diffusion***. As a band of solute molecules travels in the mobile phase, it will tend to diffuse in all directions, attributed to the concentration gradient in the column. Thus, analyte diffusion along the travel direction of the mobile phase in the chromatographic column will lead to peak broadening. To reduce the longitudinal diffusion the mobile phase velocity will set to a reasonable value. The term B (longitudinal diffusion) in the van Deemter equation is described by:

$$B = 2\,\gamma\,D_m \tag{2.13}$$

In Equation 2.13, D_m is the diffusion coefficient of the analyte in the mobile phase and δ is an obstruction factor, which describes the obstruction of the free longitudinal diffusion due to collisions with particles of the stationary phase [38, 42].

Term C is related to the ***resistance to mass transfer***. The analyte molecules should be able to partition between the stationary and mobile phases in order for an analyte to be retained. Accordingly, this implies two processes. First, ***resistance to mass transfer in the mobile phase C_m***: The analyte molecules are diffusing continuously from the mobile phase to the stationary phase and back again during their travel through the column. This transfer process is not immediate; a limited time is required for solutes to diffuse through the mobile

phase in order to access the interface and enter the stationary phase [49]. This term is given by:

$$C_m u = \frac{f(k') r^2}{D_m} u \tag{2.14}$$

In Equation 2.14, *f (k´)* is a constant which represents a function of the retention factor, and *r* is the column radius. Term C_m emanates from mass transfer in the mobile phase, which is the first part of the *C* term in the van Deemter equation.

Second, ***resistance to mass transfer in the stationary phase*** C_S: Once more, the analytes are in contact with the stationary phase and may leave and reenter the mobile phase by diffusion. Before reentering the mobile phase, the analytes have a more or less dispersed way through the stationary phase and, therefore, varying distances for back diffusion to the surface on the stationary phase. The term C_s is given by:

$$C_s u = \frac{f(k') d_f^2}{D_s} u \tag{2.15}$$

Here, d_f is the thickness of the film of stationary coated on the support and D_s is the diffusion coefficient of the analyte in the stationary phase. Term C_s arises from mass transfer in the stationary phase, which is the second part of the *C* term in the van Deemter equation. The total resistance to mass transfer *C* is:

$$Cu = C_m u + C_s u \tag{2.16}$$

This phenomenon of eddy diffusion, longitudinal diffusion and resistance to mass transfer are pictured in Figure 2.2. A typical graphic of the plate high *H* versus the average linear velocity of the mobile phase *u* in the column is shown in Figure 2.2.

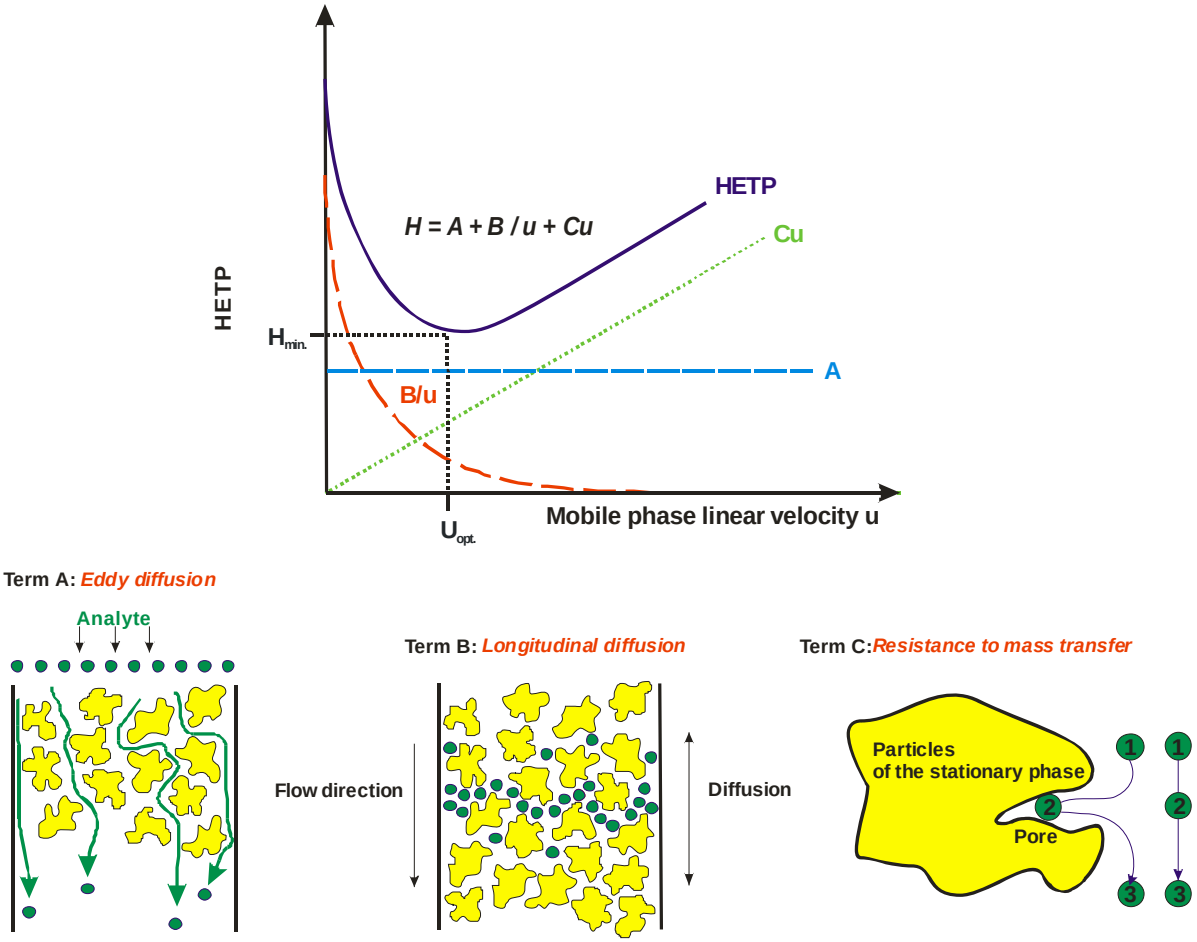


Figure 2.2: General illustration of a Van Deemter curve with representation of the individual terms A, B and C [50].

2.3 High performance liquid chromatography (HPLC)

The separation of the solute molecules that pass through the column due to the different distribution of the solutes between the liquid mobile phase and the stationary phase is a technique called liquid chromatography LC. There are two main types of liquid chromatography, classical and high-performance liquid chromatography (HPLC). Early classical liquid chromatography used columns with almost 2 cm internal diameter x 50 cm length.

The columns were filled with porous particles 50-250 µm in diameter and they often required sample quantities of milliliters. The mobile phase is usually operated by gravity at low flow rates and separation times can be in the order of hours [51].

The development of HPLC occurred in the 1970's as a powerful instrument technology in order to overcome the higher back pressures that occur when smaller particles are used for the packing. HPLC uses materials made of porous particles with diameters from 3-10 µm. These small particles are packed into columns of 100-250 mm length and 4 to 4.6 mm internal diameter. While LC consists only of a solvent reservoir and a column, the HPLC requires a complex apparatus. A typical HPLC system consists of the following components:

- High-performance pump (continuous flow at high pressure).
- Injector for sample introduction.
- Chromatographic separation column.
- Detection system with data processing.

HPLC can be used for separating and determining different substances of inorganic, organic, and biological origin. The types of HPLC are classified according to the type of stationary phase and the separation mechanism used: partition, adsorption, ion exchange, affinity, size-exclusion chromatography. The benefits of HPLC in comparison to the classical LC are of higher efficiency, shorter analysis times, higher sensitivity, better repro-ducibility and the more continuous operation [52].

2.4 Ion chromatography

Ion chromatography (IC) is a method of HPLC where ionic solute molecules are separated on positively or negatively (or both) charged sites of the stationary phase. IC is defined according to IUPAC "Separation is fundamentally based on variations in the ion exchange affinities of the solute components and usually utilizing conductivity or UV detectors"[53, 54]. In

1975, IC was introduced by *Small et al.* [55] as a new method for separating anions and cations using a conductivity detector. Ion chromatography is a generic term for three different chromatographic separation methods which are based on different separation mechanisms. We distinguish between ion exchange, ion-pair and ion-exclusion chromatography.

2.4.1 Separation mechanism of ion-exchange chromatography

In ion exchange chromatography (IEC), the solute ions are separated by ion exchange processes at the stationary phase. The separation mechanism is based on an equilibrium between charged counter ions in the mobile phase and analyte ions and the oppositely charged functional groups of the stationary phase, which can bind ions due to electrostatic forces. Generally, in anion exchange chromatography a quaternary ammonium ion is often used as the functional group of the stationary phase; in cation exchange chromatography a sulfonic acid group is used in the majority of cases. These functional groups are immobilized on support materials. Even though inorganic materials such as silica materials have been used, the polystyrene/divinylbenzene (PS/DVB) resins have become increasingly important due to their chemical stability at extreme pH-values [44]. The schematic diagram of an ion exchange process is shown in Figure 2.3.

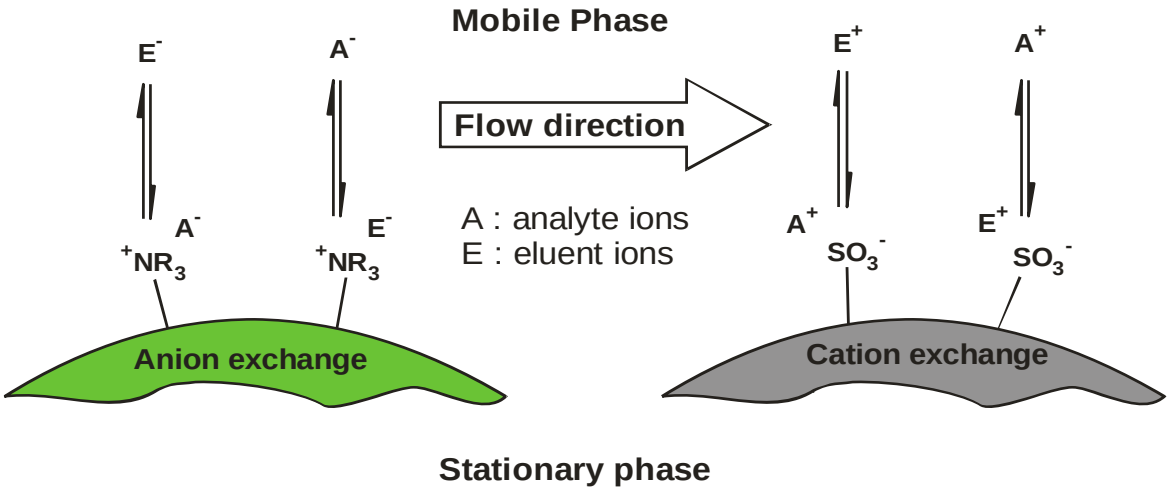


Figure 2.3: Schematic diagram of an ion exchange process [44].

If a sample containing two solute cations (A^+ and B^+) is passed through a cation exchange column, cations are exchanged for counter ions (E^+) and are retained at the fixed charge [38]. The two reversible equilibrium process are given by:

$$Resin - SO_3^- E^+ + A^+ \rightleftharpoons Resin - SO_3^- A^+ + E^+ \quad (2.14)$$

$$Resin - SO_3^- E^+ + B^+ \rightleftharpoons Resin - SO_3^- B^+ + E^+ \quad (2.15)$$

The equilibrium constant K_A^E is also called the selectivity coefficient, and is defined as follows for cations A^+ and E^+:

$$K_A^E = \frac{c(Resin{-}SO_3^- A^+).c(E^+)}{c(Resin{-}SO_3^- E^+).c(A^+)} = \frac{c(A^+)_s .c(E^+)_m}{c(E^+)_s .c(A^+)_m} \quad (2.16)$$

It is possible to separate several analytes when they are sufficiently different in their affinity for the stationary phase. In Equation 2.16 If $K_A^E = 1$, then the ion-exchange resin exhibits no selectivity for cation A^+ over eluent ion E^+, meaning that the ratios of the concentrations of analyte ions in the mobile and stationary phases are equal. If $K_A^E > 1$, the stationary phase will contain a higher concentration of analyte A^+ (over E^+) than the mobile phase [43].

2.4.2 Separation mechanism of ion pair chromatography

Ion-pair chromatography (IPC) offers a useful method for ionic analyte molecules that are difficult to separate. Ionic molecules are weakly retained in the stationary phase under RP-HPLC conditions. Consequently, ionized compounds of the opposite charge are separated on RP-columns, when an ion pair reagent is added (IPR) to the mobile phase [56]. IPC is performed on RP-columns by adding an ionic reagent to the mobile phase to interact with analyte ions of opposite charge. The separation mechanism is based on variations in interac-

tion of the different ion pairs by the stationary phase [57]. The lipophilic part of the surfactant interacts with the non-polar stationary phase. The polar part of the surfactant is used as an ion exchange site.

2.4.3 Separation mechanism of Ion exclusion chromatography

The third principle of ion chromatographic separations is the ion exclusion chromatography (ICE), which was first mentioned by *Wheaton* and *Bauman* in 1953 [58]. ICE is mainly used for the separation of weak organic and inorganic acids and of alcohols, aldehydes, amino acids and carbohydrates. This method of chromatography involves the use of anion or cation exchangers for the separation of weakly ionized analytes. Figure 2.4 illustrates the process of ion exclusion for acidic (A^-) and basic (B^+) analytes. A cation exchanger with sulfonic acid functional groups is used for the separation of weakly acidic analytes (A^-) with a partial negative charge (anion exclusion), while an anion exchanger with quaternary amine functional groups can be used for the separation of weakly basic analytes (B^+) with a partial positive charge (cation exclusion) [43].

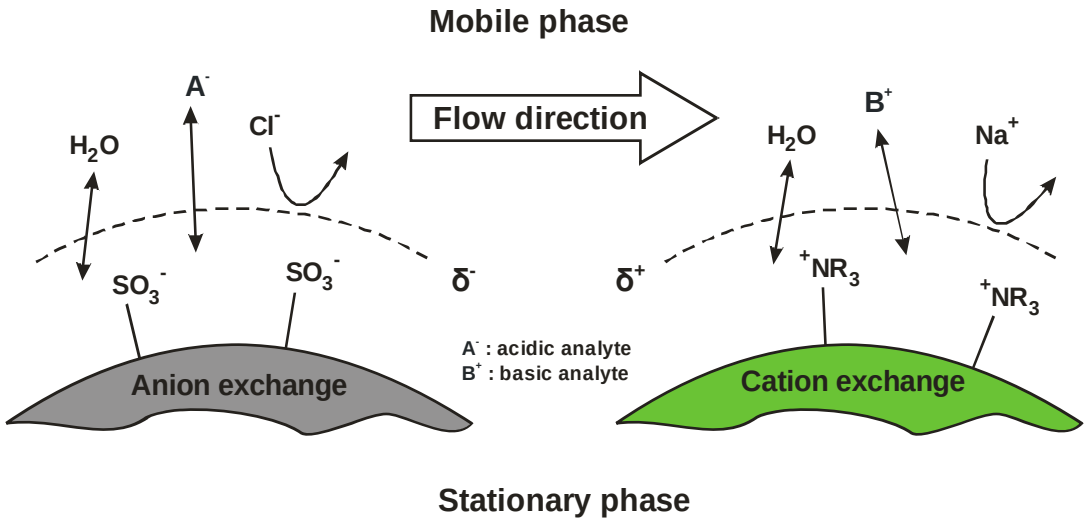


Figure 2.4: Schematic diagram of the ion exclusion process for acidic and basic analytes [43].

Once again, we observe from schema 2.4 two applications of the ion-exclusion process: Firstly, a cation exchange material used to separate a weak acid like acetic acid A^- (weak acidic) from hydrochloric acid (strong acidic) using water as the mobile phase, which shows that the Cl^- ion is repulsed by the Donnan membrane build up by the sulfonic acid groups.

Secondly, two analytes, sodium hydroxide (strong basic) and for example, ammonium B^+ (weak basic) are separated on an anion exchanger using water as the mobile phase. The permanent Na^+ ions are repulsed by the quaternary ammonia groups of the anion exchanger and are not retained by the column. Consequently, the acetic acid and ammonia are retained in the cation and anion exchangers respectively [43].

2.5 Zwitterion chromatography/hydrophilic interaction chromatography

2.5.1 Retention mechanisms of ZIC

A number of studies were published on the separation of mixtures of substances (polar and hydrophilic compounds) using zwitterionic base materials with aqueous eluents. A pioneer in this field is *Hu*, who began in 1993 to examine the effects of the zwitterion chromatography and developed first mechanistical explanations. For these investigations, RP-ODS surface materials were used, which were coated with three types of zwitterionic surfactants (inner quaternary amines and outer sulfonic acids), for the separation of inorganic anions, dansyl amino acids [3].

For a long time, water was used as mobile phase [5, 59-61] and, therefore, to solve several problems in conventional IC, for example high background signals in conductivity detection, pre-concentration of analytes. Regardless of the geometrical arrangement of the charges (Exchanger A and Exchanger B), the zwitterionic exchanger is able to separate both anions and cations as suggested in Figure 2.5.

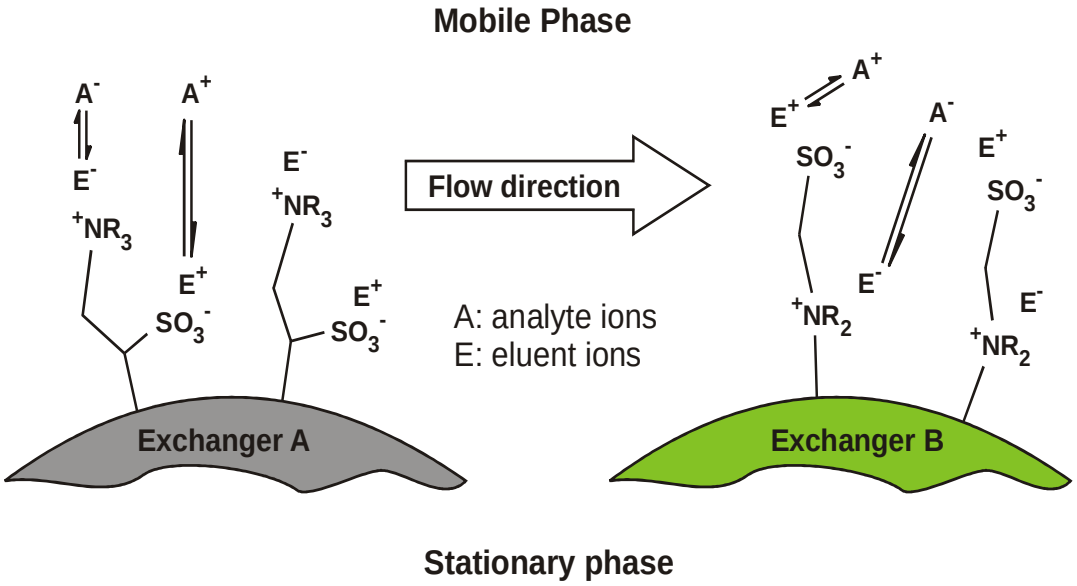


Figure 2.5: Schematic diagram of possible exchange processes in the ZIC [62].

Increasing the capacity of ZIC-exchanger means to increase the concentrations of the sulfobetaine monomer attached at the PS/DVB surface which leads to the formation of a multi-layer [11]. However, since separations can be carried out using water as the eluent as evident in the case of ZIC-exchanger, other factors must be involved in the separation. Sulfobetaine materials can form inter- or intramolecular ion pairs between the oppositely charged groups of the adsorbed sulfobetaine surfactant (Exchanger A and Exchanger B in Figure 2.6).

The steering reason for choosing either inter- or intramolecular ion pairs must be the chain length between the charged groups. With increasing spacer length, the sulfobetaine molecules on PS/DVB surface remain more flexible with increasing ability to form both intermolecular and intramolecular ion pairs [10, 11]. The reason for the necessity of the use of eluent ions in the ZIC is just the constraint of electrical neutrality by charge balance with each other charged groups on a particle.

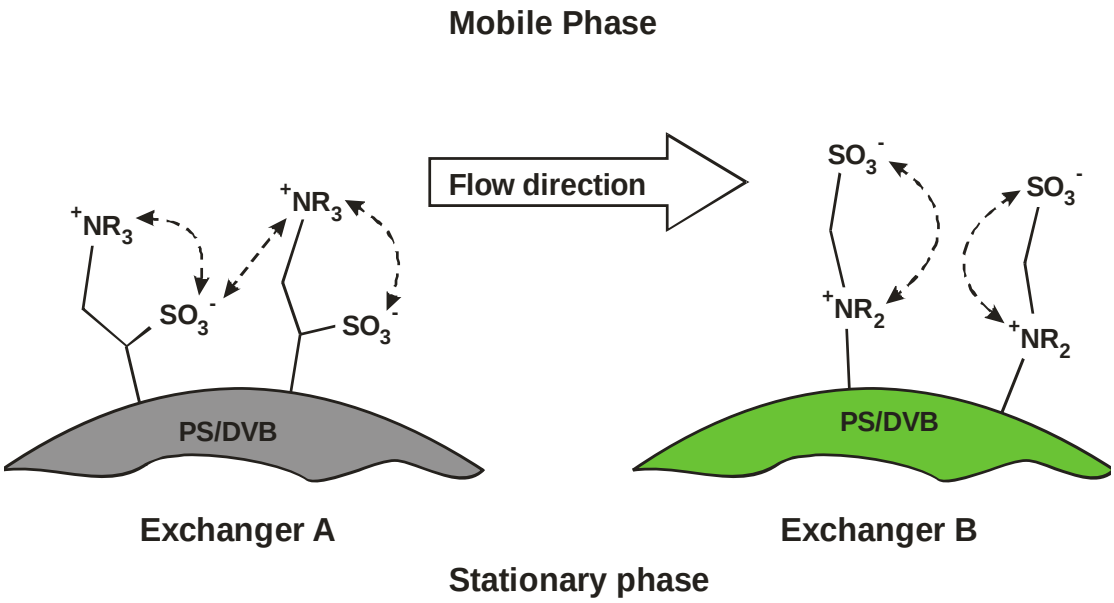


Figure 2.6: Possible structures of attached sulfobetaine on a PS/DVB surface, including inter- or intramolecular of charges [11].

A number of studies describes the separation mechanisms and retention behavior of inorganic ions on sulfobetaine stationary phases as electrostatic ion chromatography (EIC) [3, 12-14]. This mechanism includes a simultaneous electrostatic attraction and repulsion of solutes with both the positive and the negative charge of the zwitterionic stationary phase and the formation of ion pairs between oppositely charged ions.

Initially *Hu et al.* [12, 63] suggested a binary electrical double layer (binary-EDL). Both anion-EDL and cation-EDL had been formed. Consequently, the positively charged groups (quaternary amines) of zwitter- ionic surfactant stationary phase retained the inorganic anions and created an anion-EDL, while the negatively charged groups (sulfonic acids) of the zwitterionic stationary phase creating a cation-EDL when retained the inorganic cations.

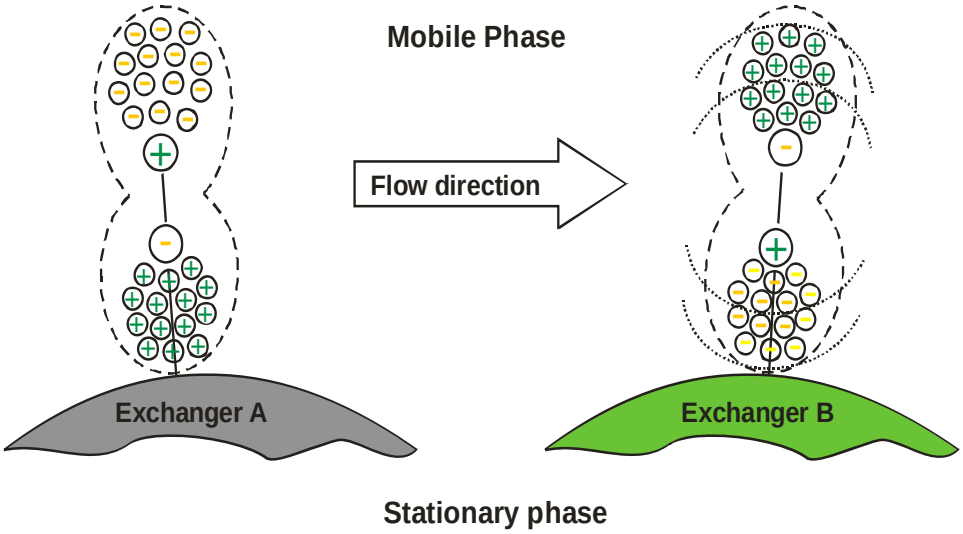


Figure 2.7: Schematic representation of the proposed the binary electrical double layer (EDL) by *Hu* [12].

The analytes (anions and cations) were retained onto the binary-EDL zwitterionic stationary phase in two manners, according to the location of positive and negative charges functional groups on the stationary phase. Firstly, in the case that zwitterionic exchanger with an outer positive charge and an inner negative charge are used as shown in Figure 2.7 (Exchanger A), solute cations should exhibit retention due to their ability to enter the binary-EDL, but solute anions exhibit no retention which is attributed to their repulsion by the anionic layer located at the upper part of the binary-EDL. Secondly, as shown in Figure 2.7 (Exchanger B), when the zwitterionic exchanger with an outer negative charge and an inner positive charge, solute anions exhibit retention due to their ability to enter into the binary-EDL, whereas cations should exhibit no retention because of repulsion by the cationic layer located at the upper part of the binary-EDL [63].

An electrolytic eluent has been shown to be useful for the separation of inorganic ions. Other effects on the separation mechanism of analytes (cation and anion) by a binary-EDL can be explained using the concept of the formation of ion-pairs. When zwitterionic exchangers with an inner negative charge are used, analyte cations were distributed into the anionic top

layer (Exchanger A in Figure 2.7) of the binary-EDL and tend to form neutral ion pairs, while, at the same time, repulsion occurs by the cationic EDL. Consequently, analyte cations, which have a high tendency to form neutral ion pairs with anions in the EDL, should exhibit strong retention. However, analyte anions with a low tendency to form ion pairs should exhibit no retention [8]. This is illustrated in Figure 2.8.

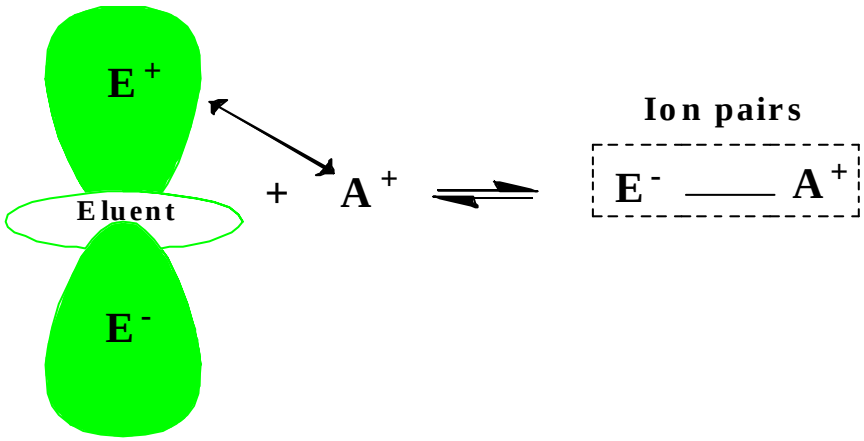


Figure.2.8: Distribution of solute cations into the anion-EDL and the formation of neutral ion pairs [63].

The influence of the eluent anion and cation on the retention of analytes was not well known and the nature of the zwitterionic exchanger has not been extensively explored. This prompted *Cook et al.* [13, 14] to offer another explanation for the zwitterionic ion chromatography retention mechanism. Their explanation is based on two simultaneous processes. Firstly, a *shield effect* is created via a repulsion of sample anions by the terminal negative charge and, therefore, the build-up of a Donnan membrane at the outer negative charge. This repulsion effect can be increased or decreased according to the interaction and shielding of the charges on the zwitterionic stationary phase by the eluent cations and anions.

Secondly, a chaotropic interaction determines the retention ability of solute ions with inner charged functional groups on the zwitterionic surfactant. Accordingly, the separation selectivity (elution order) of solute ions follows the chaotropic interactions under the Hofmeister

series [64, 65], which mean the more chaotropic a solute is, the more will be retained on the zwitterionic surfactant [14]. For anions, the interaction with the positively charged group on the zwitterionic surfactant increases according to the following series [13, 66, 67]:

$$F^-, OH^- < SO_4^{2-} < CH_3COO^- < Cl^- < NO_2^- < Br^- < NO_3^- < I^- < ClO_4^- \quad (2.29)$$

In relation to cations, shielding and retention at the negative charge on the zwitterionic surfactant increases according to the following chaotropic series [13, 68]:

$$Na^+/K^+ < NH_4^+ < Li^+ < Ba^{2+} < Ca^{2+} < (H^+) < Zn^{2+} < Ce^{3+} \quad (2.30)$$

Hydron ion is a special case, as we observe in the series above. The retention of hydron ions on the zwitterionic surfactant is very high, which means strong shielding of H^+ [69]. Figure 2.9 (a) illustrates the zwitterionic stationary phase in equilibrium with the eluent cations and anions.

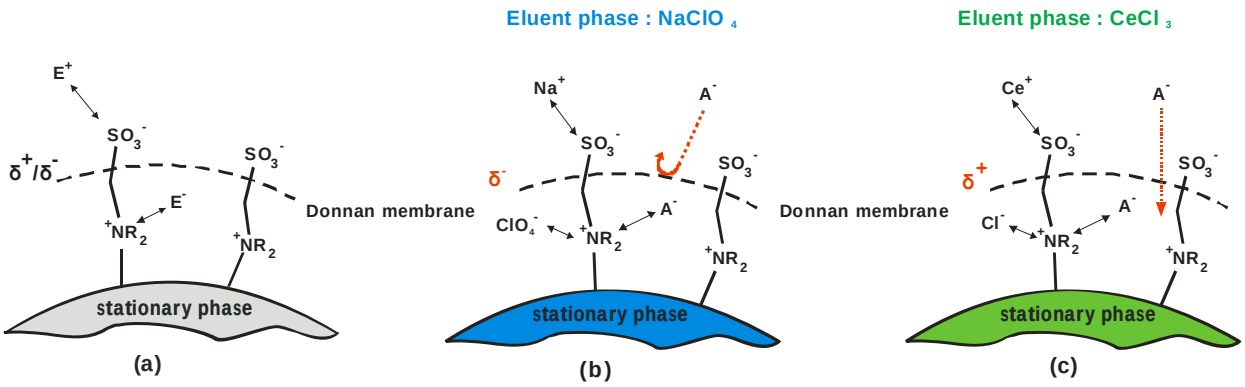


Figure 2.9: Schematic representation of the proposed Donnan membrane by Cook et al [13, 14].

For further clarification of the *Cook* mechanism, we have to discuss two cases for strong shielding of positive or negative groups on zwitterionic surfactant. The first case, as shown in Figure 2.9 b, uses $NaClO_4$ as eluent and we observe a strong interaction between ClO_4^- and the positive functional group and a weak interaction between Na^+ and the negatively charged group. Consequently, the shielding of the inner positive group should be increased, which should lead to a weak positive or even more negative Donnan membrane. The anions are retained to variable degrees by the inner positive functional groups on zwitterionic surfactant based on chaotropic interactions [14].

Second, using $CeCl_3$ as eluent, a weak shielding of Cl^- at the positive group and a strong shielding of Ce^{3+} at the negative groups, results in a more positive Donnan membrane, as is illustrated in Figure 2.9 c. The solute anions are retained with inner positive functional groups on zwitterionic stationary phase based on chaotropic interactions in accordance with the Hofmeister series [13, 14].

2.5.2 Retention mechanism of HILIC

A significantly different story compared to zwitterion chromatography (ZIC) is hydrophilic interaction liquid chromatography (HILIC). ZIC and HILIC usually differ in three factors. First, inorganic ions cannot be counted of the classical-HILIC analyte like ZIC, but of the organic (charged or uncharged and polar or small polar) compounds. Second, the mobile phase used in HILIC requires high concentrations of organic solvents, while the opposite situation occurs in ZIC. Third, the separation mechanism used in HILIC differs from that in ZIC, which will be discussed in the subsequent paragraphs.

The separation mechanism of solutes in HPLC was the subject of numerous publications. There are basically several possible models of separation principles applicable to HPLC: partitioning [70, 71], adsorption [72, 73] and retention mechanism comprising two simultaneous effects, e. g. mixed adsorption and partitioning [74]. HILIC is the alternative to normal phase liquid chromatography (NP-LC).

However, the mechanism used in HILIC is more complicated than that in NP-LC. For the mobile phase in HILIC, the solvents used resemble typical solvents for the reversed phase liquid chromatography (RP-LC) [15]. Figure 2.10 illustrates how HILIC is located in the triangle of NP-LC, IC and RP-LC.

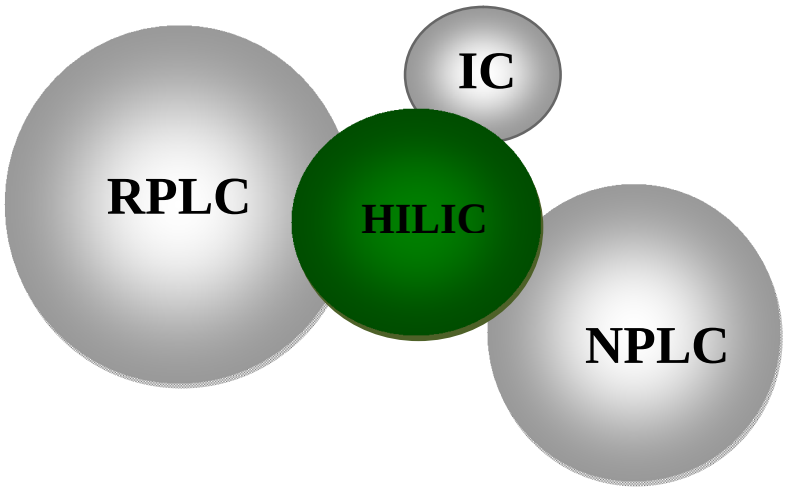


Figure 2.10: HILIC complements other areas of liquid chromatography [15].

In 1990, *Alpert* first coined the term HILIC [17]. The separation mechanism in HILIC is based on the partitioning of analyte molecules between a water-enriched layer of semi immobilized water on a hydrophilic stationary phase and a solvent-rich mobile phase [17], as illustrated in Figure 2.11. The separation is based on the hydrophilicity of analyte molecules, the more polarity of the analytes the stronger interaction with the water-rich layer on the stationary phase [15].

HILIC has many benefits over conventional NP-LC and RP-LC. For instance, the organic solvents used in HILIC mobile phases are more appropriate with mass spectrometry (MS) and are helpful to increase ESI-MS sensitivity [75, 76]. Furthermore, its ability to separate analytes in a mixture that always elute near the hold-up time in RP-columns.

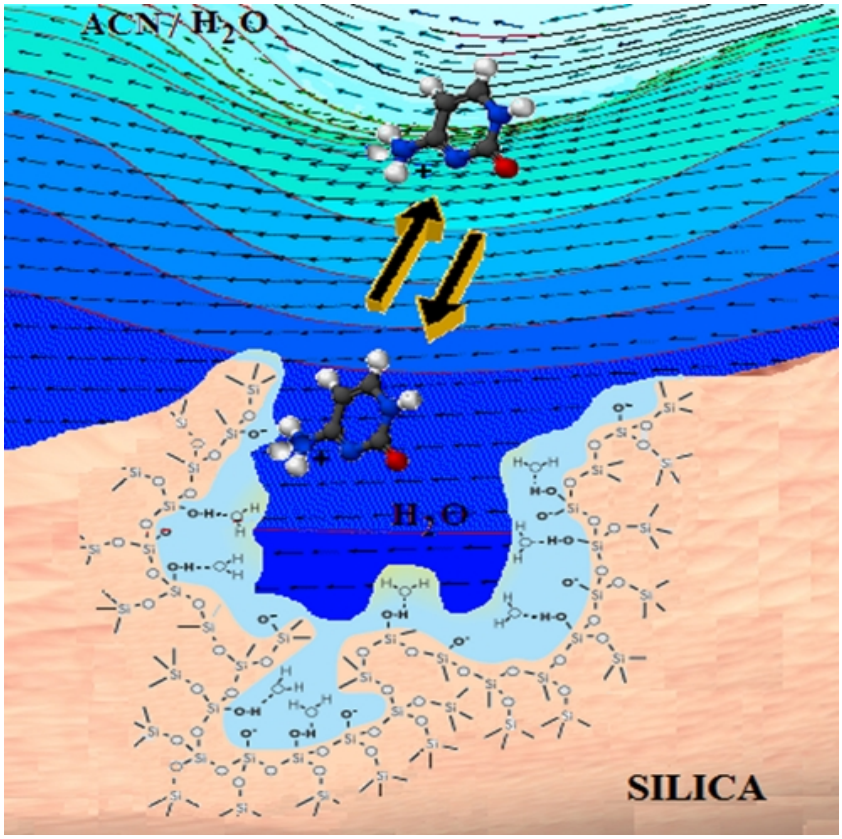


Figure 2.11: Schema of the separation mechanism in a HILIC system [15].

It is helpful to describe the influence of the concentration of eluent on the retention in HILIC modes using several equations for this purpose [77]. In ***RP-chromatography*** with binary aqueous–organic mobile phases, the retention factor of analytes decreases with increasing volume fraction φ of the stronger (organic) solvent in the mobile phase and can be described by the empirical equation [78-80]:

$$logk = logk_0 - m.\varphi = a - m.\varphi \qquad (2.22)$$

Here, *log* k_0 is the logarithm of the retention factor of the analyte in pure water, *m* is a constant decreasing with the increasing polarity of the organic solvent and with decreasing size of the sample molecule.

Equation 2.22 was supposed to be implemented in the chromatographic systems where nonlocalized adsorption controls the retention [77]. Occasionally, it is applied to HILIC systems. The retention in HILIC is inversely proportional to the polarity of the eluent and directly proportional to the polarity of the analyte molecule. Consequently, the concentration of water φ rather than the volume fraction of the organic solvent is used in Equation 2.22, in order to describe the influences of the eluent on the HILIC retention [81].

In ***NP-chromatography***, the NP retention models depend on surface adsorption, Equation 2.23 was accurately describing the NP retention models [82, 83]:

$$logk = logk_0 - m_1.log\varphi \tag{2.23}$$

The constant m_1 is the solvent elution strength factor, characterizing the influence of the concentration of the polar solvent on the rate of decrease in retention analyte molecule; k_0 is the retention factor of analyte in pure polar solvent, ϕ is the volume fraction of the more polar solvent in the binary organic mobile phase [77]**.**

2.5.3 Retention mechanisms of ZIC-HILIC

The separation mechanisms occurring in the ZIC-HILIC is more complicated. Despite the numerous studies about the ZIC-HILIC retention mode, the mechanism has not been fully investigated and there are no detailed mechanistic concepts in the literature. ZIC-HILIC is a relatively new method; this is certainly because of specific and nonspecific interactions that play a role in the mechanism. This must be attributed to the presence of charge sites in the zwitterionic stationary phase.

The ZIC-HILIC mode offers a new mixed-mode HPLC based on to the electrostatic interaction with the positively and negatively charged functional groups on the ZIC-stationary phase and hydrophilic interaction. Figure 2.13 shows the retention mechanism of analyte on the ZIC-HILIC mode. Moreover, the multipoint interactions such as hydrophilic interaction, ion-exchange interactions, hydrophobic interaction and dipole–dipole interactions contribute to the retention of analytes in ZIC-HILIC mode.

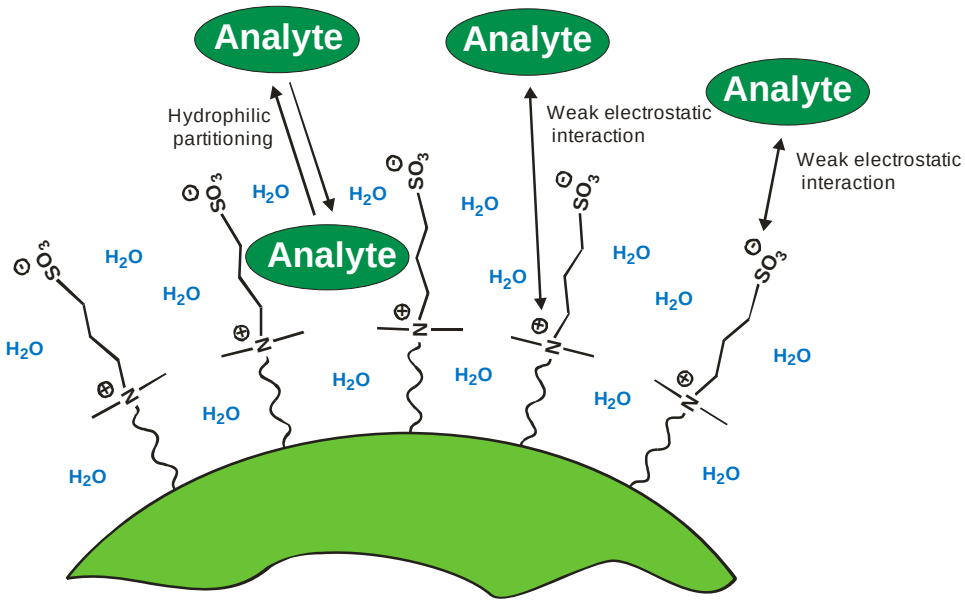


Figure 2.13: Schematic representation of the separation mechanisms in ZIC-HILIC [84].

3 Stationary phases for HILIC and ZIC-HILIC

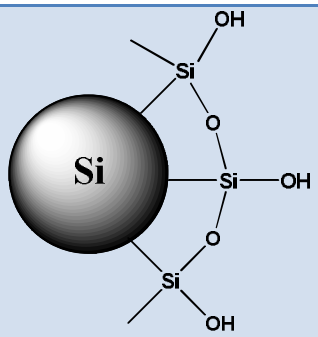
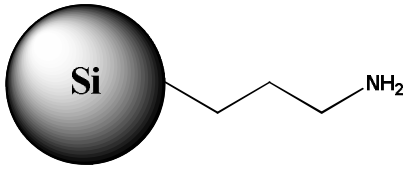
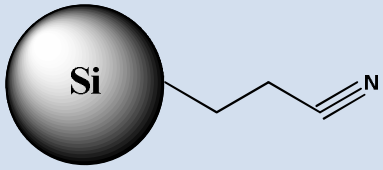
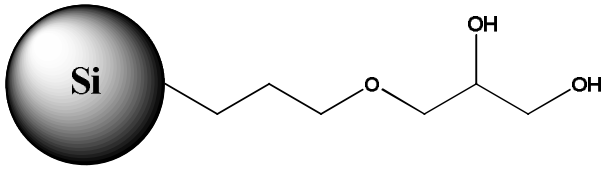
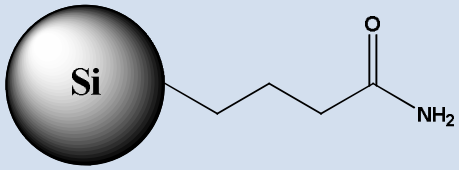
3.1 Stationary phases for HILIC

Many of the recently released publications are still using bare silica in HILIC-mode like Atlantis (Water) [85], Betasil (Thermo Hypersil) [86], Chromolith (Merk) [87], Hypersil (Ther-mo Hypersil) [88], Kromasil (EKA Chemicals) [26]. Main criteria that make bare silica attractive in liquid chromatography coupled to MS is the absence of ligands that may detach and show up as spurious peaks in the mass spectra [89]. This result in an increasing popularity of silica HILIC columns due to the widespread use of MS coupled to LC. Different types of polar stationary phase based on silica can be used in the HILIC mode for separations of biomolecules such as amino-[90], diol-[91], amide-[92], alkylamide-[93], cyano-[94], polysulf-oethyl-silica based [17]. Publications describing separations have been carried out under conditions similar to those of HILIC appeared since 1975 [16]. The number of publications related to HILIC has increased strikingly, as described in the reviews written by *Hemström et al.* [89], *Jandera* [77] and *Buszewski et al.* [15].

In 1975, the first generation of HILIC mode separations begun by *Linden et al.* [16] with separations of saccharides using an amino-based silica phase. Aminopropyl-silica columns exhibit an increased rate of anomer mutarotation significantly higher than underivatized silica, which eliminates the formation of double peaks caused by the separation of anomers. However, there are some drawbacks of aminopropyl-silica such as the materials having a limited stability against aqueous eluents [95-98], which leads to irreversible adsorption of the bonded phase by self decomposition; it comes with aldehyde compounds as analytes to the formation of Schiff bases with primary amines of the stationary phase and thus to irreversible adsorption [98]. Diol groups bonded to silica stationary phases have been used for the HILIC separation of proteins [91, 99].

Chemically bonded amide phases containing carbamoyl groups based on silica gel were used by *Yoshida* for separations of peptides under the HILIC mode [100]. It is worth noting that the amide group is less reactive than the amine group, and, therefore, less affected to eluent pH and less susceptible to irreversible adsorption [89]. Amide-silica can be used even after 500 injections of the sugar samples and exhibits good recovery and stability [100].

Table 3.1: Overview of stationary phases used in HILIC separations [15, 77, 89].

Polar stationary phases	Structure of polar stationary phase bonded to silica
Bare silica	
Amino-silica	
Cyano-Silica phases	
Diol - silica	
Amide-silica	

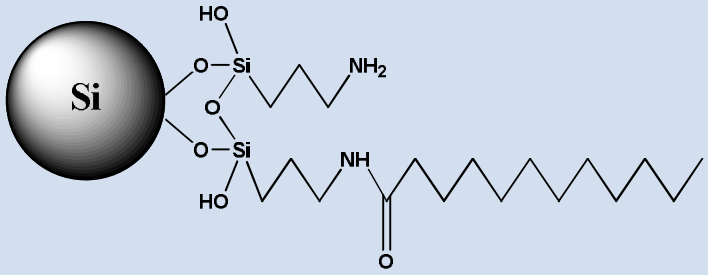
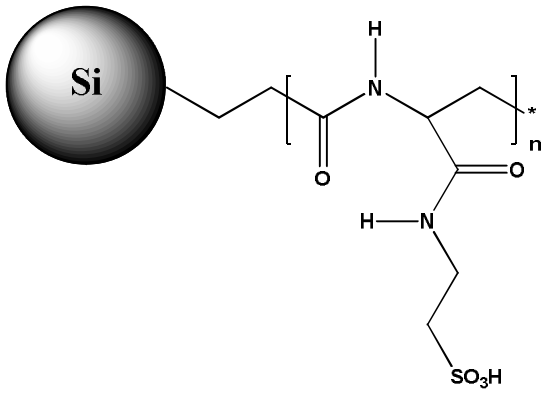
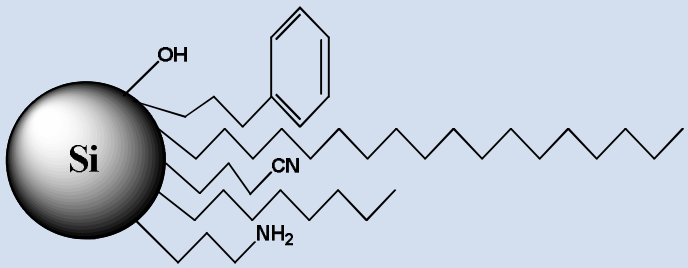
Polar stationary phases	Structure of polar stationary phase bonded to silica
Alkylamide-silica	
Poly sulfoethyl-silica	
Mix-silica ($-NH_2$, $-CN$, $-Ph$, $-C_8$, $-C_{18}$)	

Table 3.1 gives an overview of some stationary phases used in HILIC-mode separations. *Daunoravičius et al.* [101] applied cyanopropyl-silica bonded phases to the determination of denatonium benzoate, crystal violet and methylene blue in denaturated alcohol formulations. *Kowalska et al.* [102] have prepared a series of chemically bonded stationary phases with different surfaces for the examination of HILIC mode separations of nucleosides, namely aminopropyl-silica, alkylamide-silica, cholesterolic-silica and mixedstationary phase containing ($-NH_2$, $-CN$, $-Ph$, $-C_8$, $-C_{18}$).

Besides the mentioned classic HILIC-phases, in the recent 20 years zwitterionic stationary phases have increasingly been used for ZIC HILIC separations.

3.2 Zwitterionic stationary phases

Zwitterionic stationary phases are a new category of polar stationary phases used for HILIC mode separations. The distinguishing feature of zwitterionic phases is the presence of positively and negatively charged sites combined in a single molecule attached to the polymer surface [103]. Zwitterionic ion chromatography (ZIC) represents a new trend in the evolution of stationary phases for different methods of liquid chromatography. In 1951, *Stach* synthesized the first zwitterionic exchanger with inner quaternary amines and outer sulfonic acid groups [104]. The number of publications regarding ZIC has increased substantially, as outlined in the excellent reviews by *Nesterenko et al.* [2, 103].

The zwitterionic phases impart unique characteristics due to the possibility of their use for the simultaneous separation of anions and cations. According to the classification proposed by *Nesterenko et al.* [2] classifying the zwitterionic stationary phases depending on their structure and distribution of oppositely charged groups in the phase as follows: First, zwitterionic exchangers with positively and negatively charged functional groups randomly distributed within the whole volume of the polymer surface as shown in Figure 3.1 (a), e. g. a snake-cage resin was synthesized by *Hatch et al.* [105], in which the ion-exchange resin matrix constitutes a polymer “cage” copolymerized with an organic counter ion to constitute linear polymer chains of positive and negative charged polymer “snakes”. The most important advantages of snake-cage resins are a less affinity towards non-electrolytes and, therefore, desalting non-ionic solutions [2, 104].

Second, the stationary phase with positively and negatively charged functional groups separately distributed charges on the surface as shown in Figure 3.1 (b), e. g. multifunctional zwitterionic stationary phase [106, 107]. The simplest manner to produce this type is dynamica coated of reversed phase substrates with a mixture of cationic and anionic surface

tants [2]. In 1993, *Macka* and *Borák* were able to develop the material in which a mixture of a cationic (tetrabutylammonium) and an anionic (octanesulfonate) were dynamically modified to octadecylsilica (ODS) [108] for the separation of cationic, anionic and uncharged platinum (II) complexes.

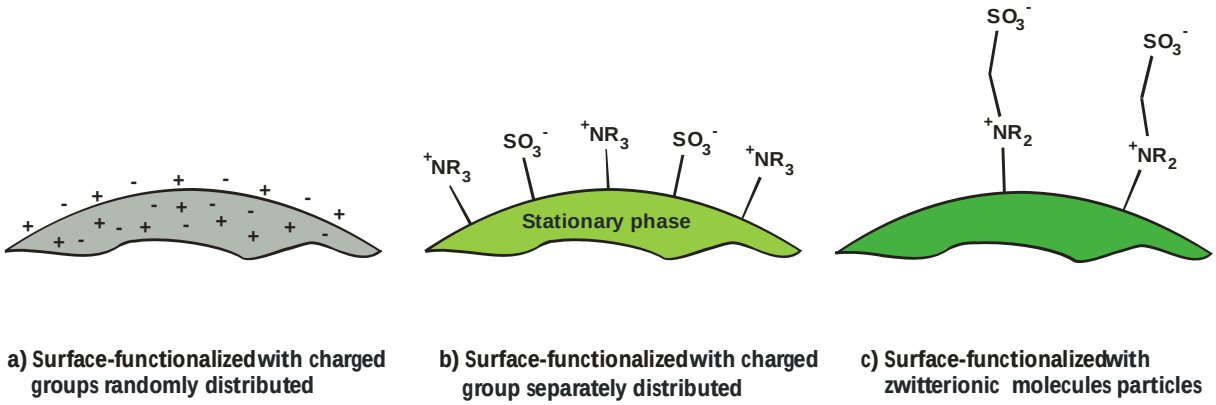


Figure 3.1: Scheme represents categories of zwitterionic stationary phases in liquid chromatography [2].

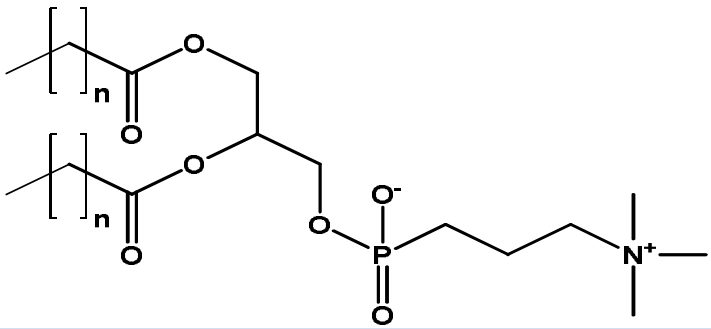
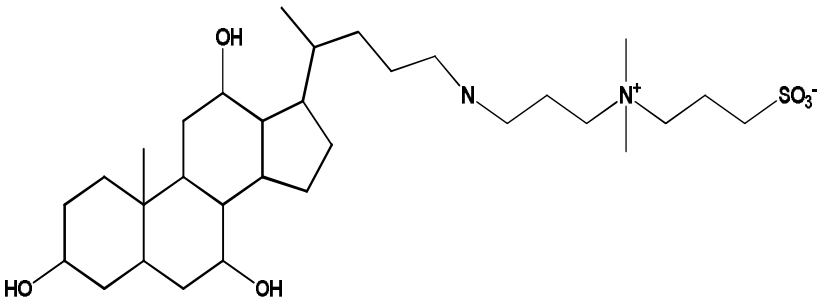
Thirdly, the stationary phases with zwitterionic molecules are present in a single molecule as illustrated in Figure 3.1 (c). There are two manners for the preparation of this type, dynamic modification and covalently attached with zwitterionic surfactants.

3.2.1 Stationary phases dynamically coated with zwitterionic molecules

To obtain zwitterionic stationary phases, the dynamic modification is one of the easiest ways to create a polymer surface with zwitterionic molecules. Essentially, the covalent binding of zwitterionic groups to support materials is a very challenging task [103]. For this reason, initial studies were performed by coating of RP materials with zwitterionic sur-

factants. In this way, a number of studies dealing with stationary phases dynamically modified with zwitterionic surfactants, such as carboxybetaine-type [11, 109], phosphocholine-type [110, 111] and sulfobetaine-type [3, 6, 63, 112, 113]. Table 3.2 shows the different structures of zwitterionic surfactants used for the preparation of dynamically coated zwitterionic stationary phases.

Table 3.2: Overview of some of the zwitterionic surfactants used for dynamic coating [2, 103].

Zwitterionic surfactants	Structure of zwitterionic surfactants
Zwittergent 3-14 N-alkyl-N,N-dimethyl-3-ammonio-1-propanesulfonate	
n = 12: Dimyristoyl-phosphadylcholine	
n = 3: N-(Dodecyl-N,N dimethylammonio)butyrate	
CHAPS 3 - [(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulfonate	

Several separation mechanism models of zwitterionic stationary phases have been proposed. For ZIC-mode *Okada et al.* [114, 115] studied mechanisms based on the Poisson–Boltzmann theory, *Hu et al.* [8, 12] proposed a retention mechanism depending on a binary-EDL model, *Cook et al.* [13, 14] suggested a Donnan membrane mechanism based on both an ion exclusion effect and the chaotropic interaction. For the ZIC-HILIC mode, analyte is distributed between the water-enriched stationary layer and the mobile phase with low water contents [17] and is also influenced by electrostatic interactions [84].

The main drawback of dynamically coated columns is the remarkable lack of stability. *Glenn and Lucy* [116] have investigated coating conditions such as surfactant concentration, acetonitrile content, ionic strength and temperature. They found the necessity of adding the surfactant to the eluent, according to this the retention time and efficiency remained stable for ≥ 3000 column volumes. For this reason, it makes sense to find possible solutions to this problem including the idea of attaching zwitterionic molecules covalently to the support materials.

3.2.2 Stationary phases covalently attached to zwitterionic molecules

In 1986, *Yu et al.* [117] synthesized the first zwitterionic stationary phase covalent bonded onto a silica core, which contain quaternary amino and sulfonic acids functional groups, for separation of a mixture of zwitterionic, anionic and cationic analytes. Thereafter, in 1989, for separation of amino acid, peptide and pharmaceutical solutes, *Yu et al.* [118] developed a covalently bonded sulfobetaine-exchange stationary phase. Further improvements were made in the stationary phases covalently bond with zwitterionic surfactants by *Irgum* and co-worker; they proposed two ways for the production of bonded zwitterionic stationary phases.

First, direct functionalization [1] based upon activated 2-hydroxyethyl methacrylate-ethylene dimethacrylate copolymer (HEMA-EDMA) with epichlorohydrin, thereafter treatment with 2-(dimethylamino) ethanesulfonic acid (DMAES), resulting in a zwitterionic stationary phase with inner quaternary amine and outer sulfonic acid functional group (Table 3.3) for simul-

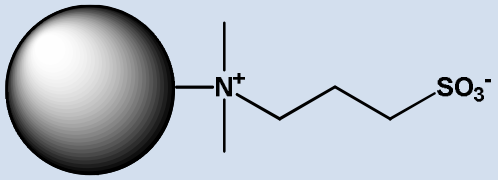
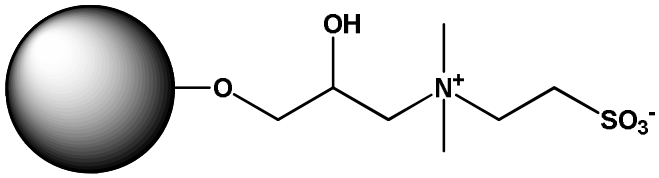
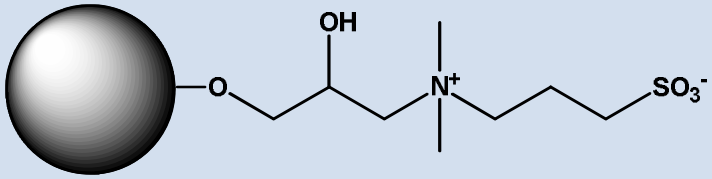
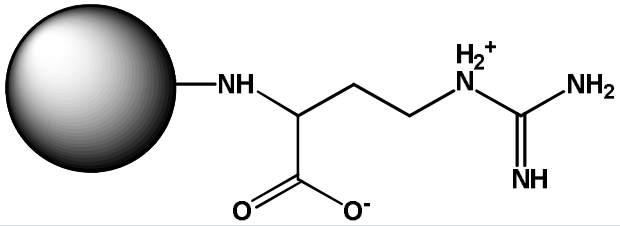
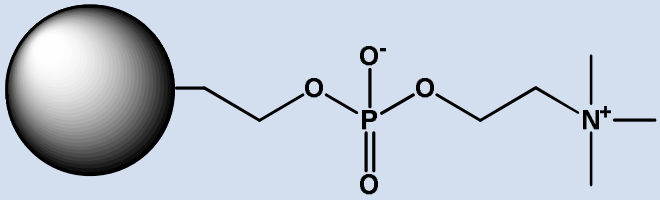
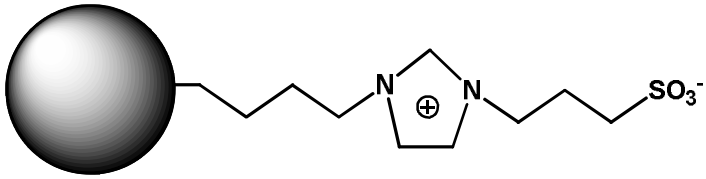
taneous separation of inorganic cations and anions using water and perchlorate as eluents. Two different zwitterionic stationary phases were also based on the activat ed hydroxyl group route using 3-[*N*,*N*-dimethyl-*N*-(methacryloyloxyethyl) ammonium] propanesulfonate and 3-dimethylaminopropanesulfonate as second step reagent resulting in the zwitterionic stationary phases S300-ECH-DMA-PS and S300-TC-DMA-PS respectively (Table 3.3) [119].

Second, graft polymerization [120] functionalization of silica particles is performed by free radical graft polymerization with 3-[*N*,*N*-dimethyl-*N*-(methacryloyloxyethyl) ammonium] propanesulfonate for the production the KS-TC-TBHP-SPE zwitterionic stationary phase. This stationary phase is able to separate acidic and basic proteins. Another way to obtain zwitterionic stationary phases is the attachment of amino acids via covalent bonding to the surface of polymers such as glutamic acid-silica [121], lysine-silica monoliths [122], arginine-HEMA, glutamine-HEMA and histidine-HEMA [123]. This type of stationary phases has benefits regarding the possibility of selectivity manipulations attributed to the variable positive and negative site capacities depending on the eluent pH [103].

In addition to the phases already mentioned, two novel zwitterionic materials for the separation of polar and non-polar compounds were designed by Sielc (Prospect Heights, IL, USA), under the trade names Obelisc N and Obelisc R. Obelisc R has reversed-phase character (with the inner anion exchange group and outer cation exchange group), Obelisc R exhibits to be more convenient for the separation of RP applications due to the of hydrophobic chain and ionic sites. Obelisc N has normal-phase character (with the inner cation exchange group and outer anion exchange group), Obelisc N shows more appropriate for separation NP applications due to a hydrophilic chain and ionic groups. This material is offered for HILIC applications [124].

Sonnenschein and Seubert [10] synthesised five new sulfobetaine molecules stationary phases based on PS/DVB with covalently bonding using a grafting reaction, resulting in zwitterionic exchangers differing in chain length between the charged functional groups; inorganic anions [10] and amino acids [125] were used as analytes.

Table 3.3: Overview of a selection of covalently bonded zwitterionic exchangers [103].

Column-zwitterionic molecules	Structure of stationary phase
ZIC-pHILIC Sulfobetaine-type attached to the methacrylate core **ZIC-HILIC** Sulfobetaine-type attached to the silica core	
HEMA–DMAES 2-(dimethylamino) ethanesolfonate attached to the poly-hydroxy-ethyl-methacrylate	
HEMA-DMAPS 3-[*N*,*N*-dimethyl-*N* (methacryloyloxyethyl) ammonium] propanesulfonate attached to poly-hydroxy-ethyl-methacrylate	
Arg-HEMA poly-hydroxy-ethyl-methacrylate with covalently attached to arginine	
ZIC-cHILIC Phosphocholine-group attached to the silica	
SiImPS 1-Alkyl-3-(propyl-3-sulfonate)-imidazolium-functionalised silica stationary phase	

Some of the common zwitterionic exchangers are summarized in Table 3.3. It is worth mentioning that small numbers of covalently bonded zwitterionic stationary phases have been studied previously. For this reason, systematic investigations on a homologous set of zwitterionic exchangers can contribute significantly to a deeper understanding of zwitterionic chromatography in ZIC and ZIC-HILIC modes.

4 Results and discussion

4.1 Synthesis of zwitterionic molecules

The focus of my work is the preparation and characterization of four zwitterionic stationary phases and their characterization using ZIC and ZIC-HILIC mode separations and the measurement of surface excess isotherms via the minor disturbance method. The synthesis of four zwitterionic precursors with inner quaternary amines and outer sulfonic acids was prepared according to a procedure published by *Sonnenschein and Seubert* [126]. However, before the chromatographic investigations can be carried out, the exchanger materials must first be prepared reproducibly and with similar properties (e. g. the core material, the spacers to the polymeric backbone) and, therefore, differing only in intercharge spacer length between the sulfobetaine materials.

Figure 4.1 illustrates the synthetic strategy for the preparation of zwitterionic stationary phases via two steps. The first step is the synthesis of the zwitterionic molecules, which is already bearing the functional groups and provide a way of connecting to the base material. Thereafter, the second step is graft polymerization, in which the connection of the zwitterionic molecules to the PS/DVB carrier particles is achieved by radical addition.

The individual reactions of the first step for the preparation of zwitterionic monomers with different distances between the charged functional groups include sub-steps. Zwitterionic monomers with one to two methylene groups between the charges were prepared using molecules containing tertiary amines and the sulfonic acid functionality in the first sub-step and the attachment of these molecules to the spacer (4-vinylbenzylic chloride) in the second step using a grafting reaction (Figure 4.1).

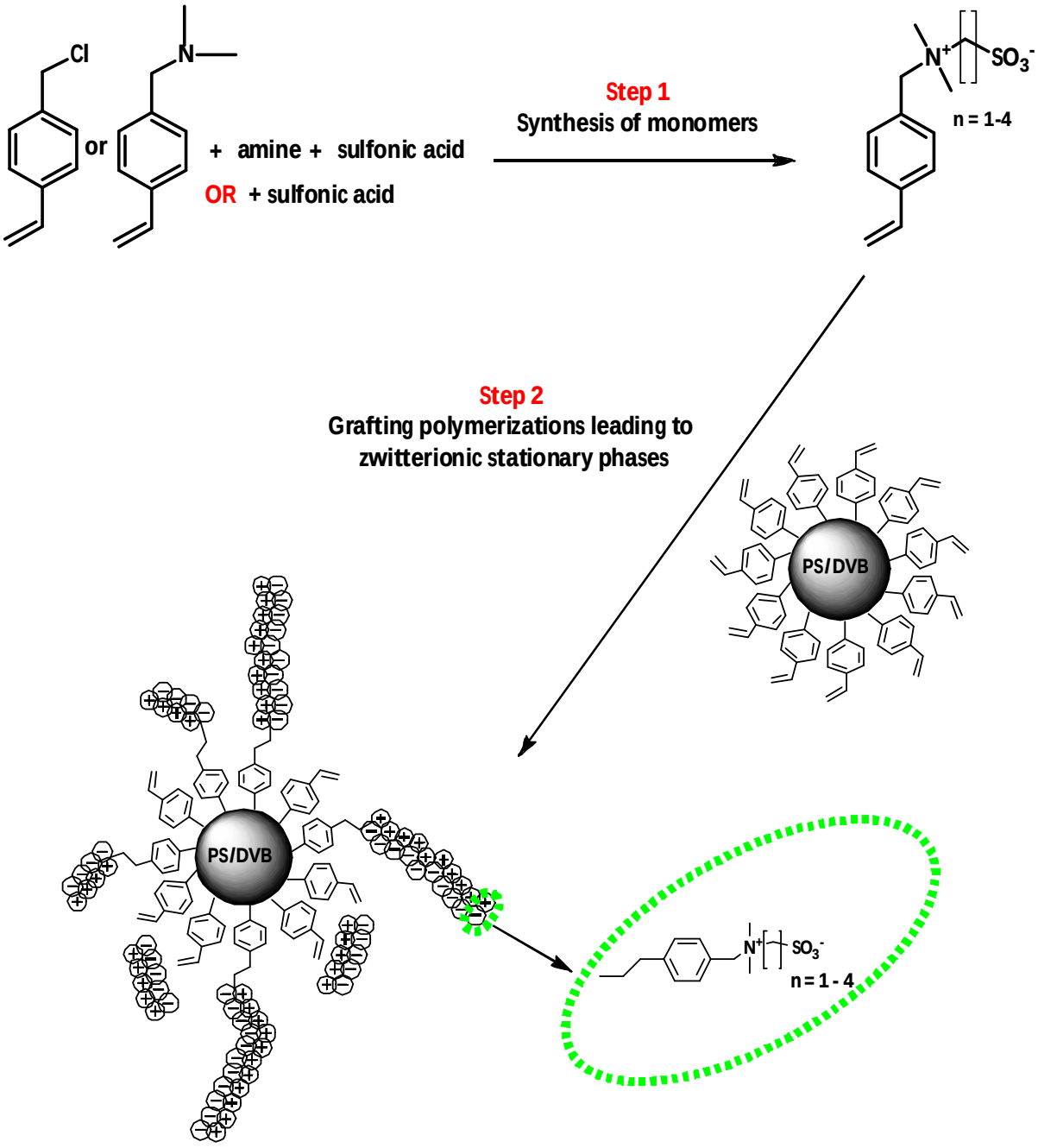


Figure 4.1: Schematic reaction sequence of the preparation of zwitterionic stationary phases.

Zwitterionic monomers with three to four methylene groups between the charged functional groups were prepared using a tertiary amine (4-vinylbenzyl-*N, N*-dimethylamine) attached to a sulfonic acid molecule. Afterwards, the product was attached to the PS/DVB core material via grafting reaction (Figure 4.1). The structures of the zwitterionic monomers (SB1, SB2, SB3 and SB4) are summarized in Figure 4.2.

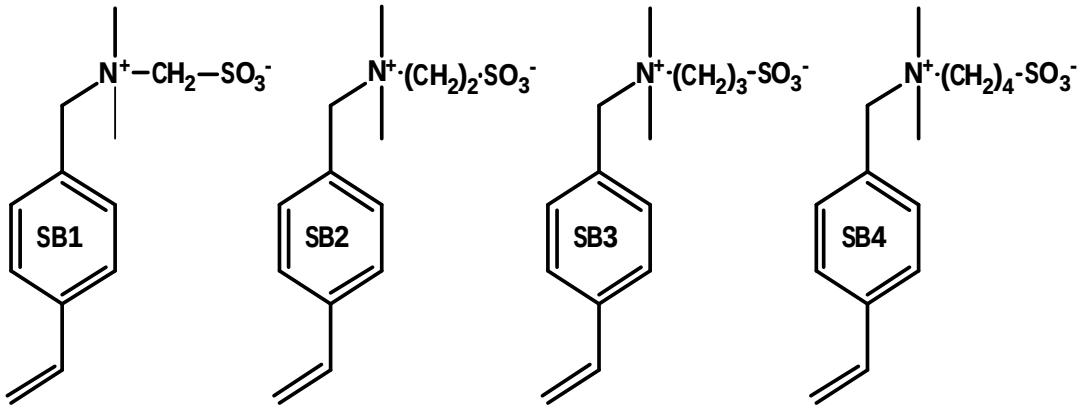


Figure 4.2: Overview of the synthesized sulfobetaine monomers SB1 to SB4.

The connection of the monomers proceeds via a surface functionalization of the substrate by radical addition. Functionalization of the PS/DVB is implemented by a grafting reaction following a recipe developed by *Raskop et al.* [127].

4.1.1 Preparation of sulfobetaine monomers SB1 and SB2

Syntheses of zwitterionic precursors were achieved by two sequential nucleophilic substitution reactions for sulfobetaines SB1 and SB2 [126]. The nucleophilic substitution reactions play a crucial role in the entire synthesis process of the sulfobetaine (SB1) having one methylene group between the charges.

The preparation is carried out according to a procedure published by *King and Skonieczny* [128] as shown in Figure 4.3. According to this procedure, *the first stage of the reaction*, the sodium hydroxyl methanesulfonate, is applied ice cooled in aqueous solution. Afterwards, through slow dropwise addition of *N,N*-dimethylamine (40% in water) a nucleophilic substitution of the alcohol function (hydroxyl group) by the secondary amine takes place. After the addition of *N,N*-dimethylamine, the ice bath is removed and the solution stirred for one hour at room temperature, which leads finally to the tertiary amine (*N,N*-dimethylamino methanesulfonate) [126]. The product isolated as a colorless powder was characterized by NMR spectroscopy.

The nucleophilic substitution of the *second reaction step* is carried out under nitrogen amosphere as the precursor of this reaction is a styrene derivative and thus capable of polymerization. In this case, the intermediate product (*N,N*-dimethylamino methanesulfonate) dissolved in ethanol/water (1:1) is added dropwise to 4-vinylbenzyl chloride in ethanol under ice cooling.

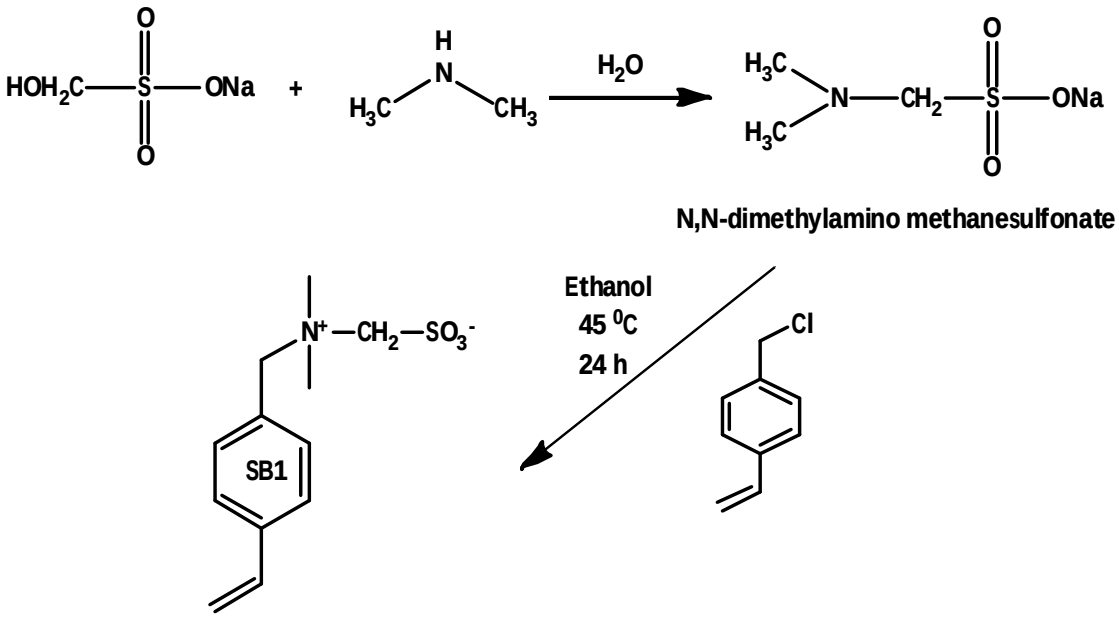


Figure 4.3: Synthesis of 4-vinylbenzyl-dimethylammonio methanesulfonate SB1 [126].

In this reaction, the ice cooling removes the excessive heat generated by the initial meeting of the reactants. After removing the solvent under reduced pressure, the desired SB1-

monomer (4-vinylbenzyl-dimethylammonio methanesulfonate) is obtained as a colorless solid, which can be examined via elemental analysis and NMR spectroscopy.

In contrast to the first reaction stage of the SB1 synthesis, the nucleophilic substitution of the chlorine atom of the 4-vinylbenzyl chloride by the tertiary amine of N,N-dimethylamino methanesulfonate is slower. This behavior is due to the higher steric hindrance of the tertiary amine as opposed to the secondary amine in the first stage reaction.

In comparison to the preparation of the SB2 monomer (4-vinylbenzyl-dimethylammonio ethanesulfonate), proceeds via different way (Figure 4.4) starting form tertiary amine 2-chloro-N,N-dimetylethylamine hydrochloride. *The first reaction step* for the synthesis of the SB2 monomer is carried out according to a procedure by *Palmi et al.* [129] via reaction of 2-Chloro-*N,N*-dimethylamine hydrochloride with sodium metabisulfite into the aqueous solution and boiling for four hours. Afterwards the reaction mixture is treated with a strong acidic cation exchanger (Lewatit S) in the H^+-form in order to remove the excess sodium metabisulfite. By washing the cation exchanger with acetic acid and subsequent removal of the acetic acid by vacuum the *N,N*-dimethyltaurine hydrochloride can be isolated as sodium salt.

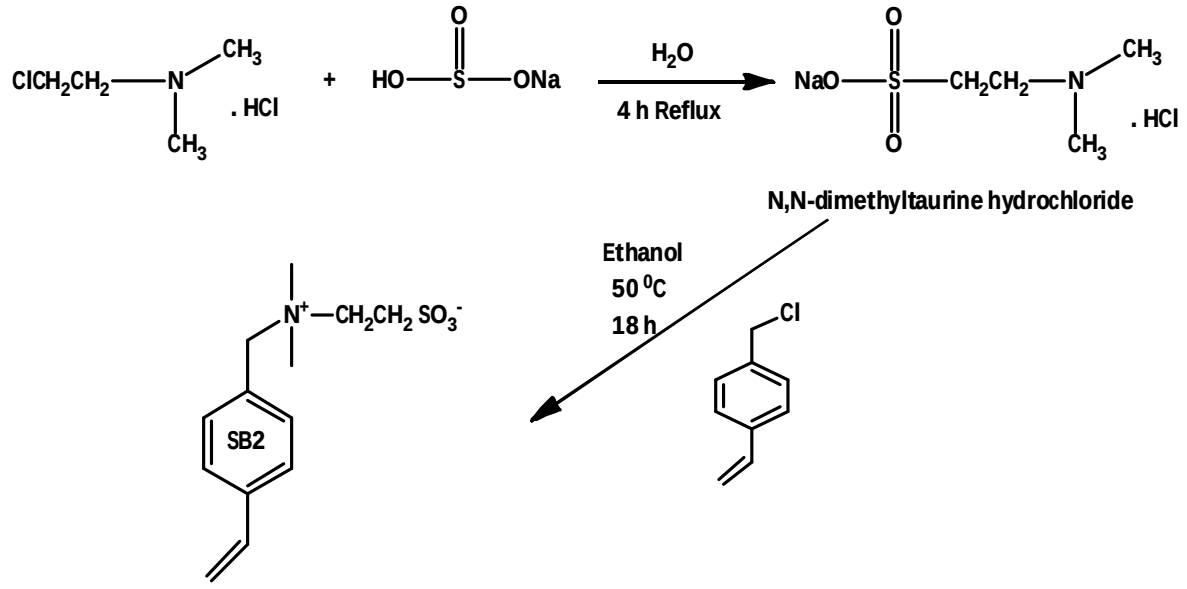


Figure 4.4: Synthesis of 4-vinylbenzyl-dimethylammonio ethanesulfonate SB2 [126].

The hydrochlorides of amines usually had better water solubility than the free amines, but they are also significantly less nucleophilic. This is especially problematic when used in the nucleophilic substitution of the chlorine atom of 4-vinylbenzyl chloride (second step). To overcome this problem, the free amine must be present by adding ammonium hydroxide. Ammonium hydroxide has to be added to the mixture to increase reaction rates due to the reduced reactivity of hydrochlorides of amines [126]. Subsequently, the reaction mixture was heated for 18 hours at 323 k. After removing the solvents under reduced pressure, the product obtained was washed with acetonitrile and finally 4-vinylbenzyl-dimethylammonio ethanesulfonate (SB2) was isolated as colorless powder.

4.1.2 Preparation of sulfobetaine monomers SB3 and SB4

The sulfobetaine molecules SB3 (4-vinylbenzyl-dimethylammonio propansulfonat) and SB4 (4-vinylbenzyl-dimethylammonio butanesulfonate) are synthesized in a slightly different way by nucleophilic reaction [126]. In contrast to the previously described syntheses, the reaction involves only one-step and the sulfonic acid is a cyclic compound (sultone) as shown in Figure 4.5. The sulfobetaine monomers SB3 and SB4 were synthesized based on a polymer analogous reaction step described by *Jiang* and *Irgum* [119], which has been previously discussed in chapter 3. The reactants 4-vinylbenzyl-*N,N*-dimethyl amine and 1,3-propane- sultone or 1,4-butanesultone are dissolved in acetonitrile and heated for 48 hours at 323 k. The SB3 and SB4 monomers are obtained as a colorless precipitate. The nucleophilic substitution by the tertiary amines (4-vinylbenzyl-*N,N*-dimethylamine) results in ring opening of the sultone and leads to the formation of both, anion and cation exchange sites.

4.2 Investigations of the zwitterionic monomer

For further characterization of the sulfobetaine monomers SB1 to SB4, various analytical methods have been applied. NMR studies were generally used in the synthesis in order to ensure the structure and the purity of the sulfobetaine molecules that have been produced.

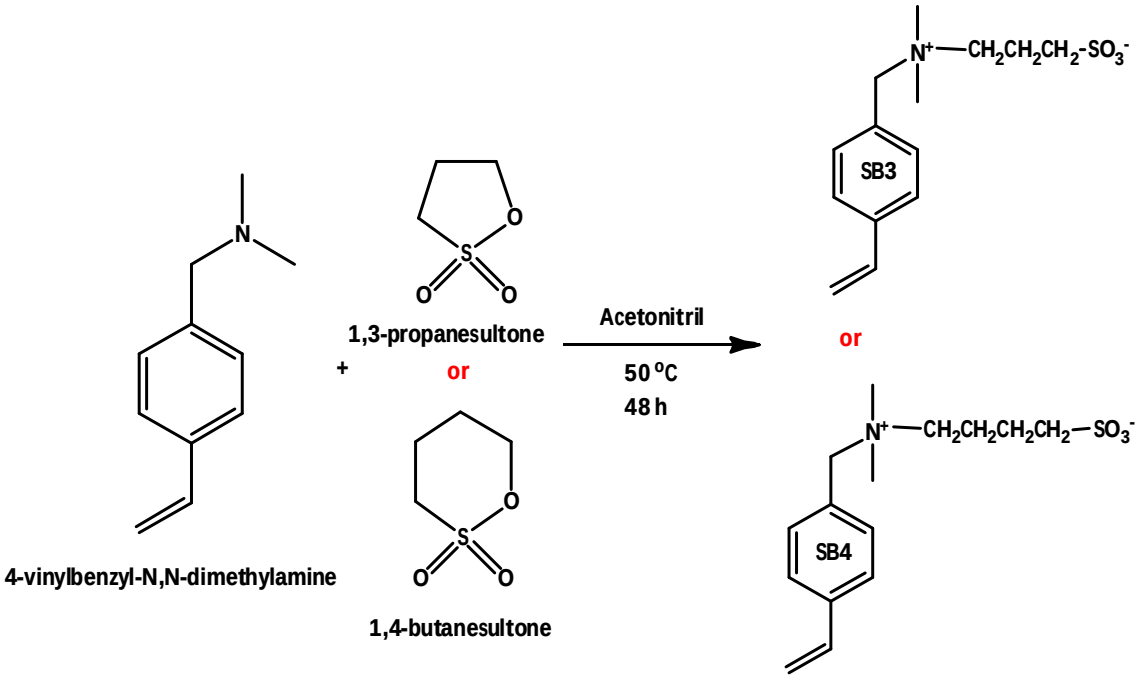


Figure 4.5: Synthesis of sulfobetaine monomers with three to four methylene groups between the charge functional groups [126].

Several studies have been conducted on the sulfobetaine monomers by *Sonnenschein et.al.* [62] to answer many questions about the structure of the monomers, such as the counter ion of charged functional groups (quaternary amines and sulfonic acids) which is required for electrical neutrality and for the correct molecular mass of the monomers in order to use the radical initiator in the right stoichiometric concentration in the graft polymerizations.

Table 4.1 illustrates the molecular mass and formula of the sulfobetaine monomers and exhibits that SB1 and SB2 exchangers have sodium chloride as a counter ion; Na^+ ion as a counter ion for sulfonic acid, Cl^- ion as counter ion for the amine. The SB3 and SB4 exchangers do not have counter ions as the charges are compensated intramolecularly. They form so-called internal ion pairs (Figure 4.6).

Table 4.1: Molecular mass and molecular formulas of the sulfobetaine monomer produced [62].

Sulfobetaine monomer	Molecular mass / g mol^{-1}	Molecular formula
SB1	313.78	$C_{12}H_{17}ClNNaO_3S$
SB2	327.80	$C_{13}H_{19}ClNNaO_3S$
SB3	283.39	$C_{14}H_{21}NO_3S$
SB4	297.41	$C_{15}H_{23}NO_3S$

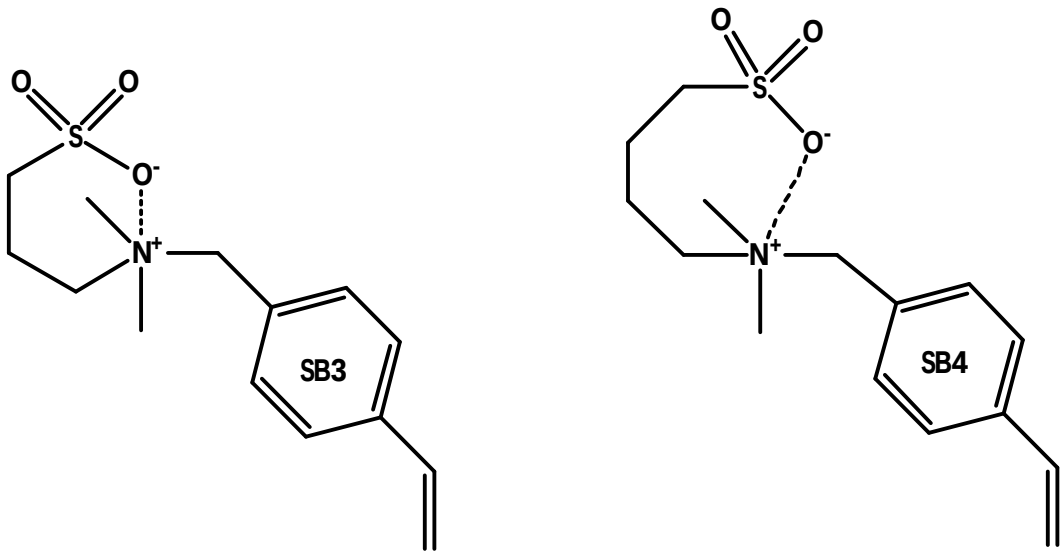


Figure 4.6: Schematic illustration of a conceivable internal ion pair of sulfobetaine molecules SB3 and SB4 [62].

Another interesting characteristic of zwitterionic monomers is the hydrophobicity of the prepared zwitterionic molecules. In a previous study, *Sonnenschein et al.* [10] investigated the polarities of zwitterionic monomers by RP-HPLC, they explained that the polarity should be dependent on the ability of the monomers to compensate their charges via intramolecular interaction.

They found that the SB2 material showed the highest polarity compared to SB1, SB3 and SB4 exchangers (Figure 4.7). The reason for this must be the chain length between the charged groups. With increasing spacer length, the sulfobetaine molecules remain flexible on RP surfaces with increasing ability to form both intermolecular and intramolecular interactions. The shortest chain molecule SB1 exhibited the lowest polarity compared to SB material due to the SB1 having an inflexible geometry, which get the charges compensated intramolecularly.

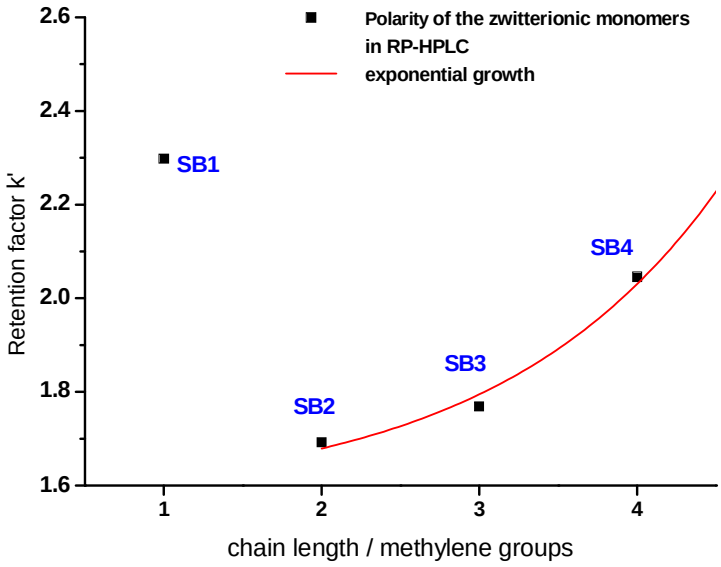


Figure 4.7: The retention factors of the four zwitterionic monomers against the distances between the charges in methyl groups [10].

4.3 Synthesis of stationary phases for zwitterion chromatography

4.3.1 Graft polymerization of zwitterionic monomers

The graft polymerization is carried out according to the method developed by *Raskop et al*. [127, 130] (method shortcut is EVO-III). A sketch of the method is shown in Figure 4.1. Initially, the PS/DVB was preheated in a solvent mixture of acetone/water (20:80). After a certain time, the sulfobetaine monomer was added and the solution was stirred for 15 minutes. Thereafter, the radical initiator was added in one portion as a solid. The reaction time was generally 4 hours; the reaction temperature was 333K. Detailed reaction conditions were given in paragraph 5.3.1.2 of the experimental part.

The stationary phases were packed into PEEK columns (100 mm × 4 mm I.D.) using a down fill slurry technique [43] with the functionalized material suspended in acetate buffer (100 mM, pH 4.75) and the same acetate buffer as packing fluid. For fast and dense packing, a pressure of 500 bar was applied. For the production of two separation columns (for ZIC and ZIC-HILIH), 3 g of functionalized polymer were needed.

4.3.2 Nomenclature of the prepared zwitterionic stationary phases

All prepared columns are labeled SBX-Y-Z. The letter prefix SB indicates the functionality of sulfobetaine. The number X refers to the chain length between the charges of each monomer, so SB1 stands for the monomer with one methyl group between the quaternary amine and sulfonic acid. The letter Y refers to the concentration step of the monomer and should reflect the capacity.

The letter Z refers to the number of the column (as a rule, the numerals 1 and 2 indicate the column number used in ZIC and ZIC-HILIC modes, respectively). Table 4.2 illustrates the nomenclature of prepared columns of each sulfobetaine monomer by varying different amounts of the sulfobetaine molecules to produce different capacities via the graft polymerization.

Table 4.2: Manufactured zwitterionic exchanger materials via the EVO-synthesis.

Column	Monomer	Monomer amount	EVO-Condition
		4 mm-Column, 3 g of PS-DVB polymer	
SB1-A-1	**SB1**	**1.80 mmol**	**333K, Acetone/water (20:80)**
SB1-A-2	SB1	1.80 mmol	333K, Acetone/water (20:80)
SB1-B-1	SB1	2.79 mmol	333K, Acetone/water (20:80)
SB1-B-2	SB1	2.79 mmol	333K, Acetone/water (20:80)
SB1-C-1	SB1	3.93 mmol	333K, Acetone/water (20:80)
SB1-C-2	SB1	3.93 mmol	333K, Acetone/water (20:80)
SB1-D-1	SB1	4.98 mmol	333K, Acetone/water (20:80)
SB1-D-2	SB1	4.98 mmol	333K, Acetone/water (20:80)
SB1-E-1	SB1	7.50 mmol	333K, Acetone/water (20:80)
SB1-E-2	SB1	7.50 mmol	333K, Acetone/water (20:80)
SB2-A-1	**SB2**	**3.71 mmol**	**333K, Acetone/water (20:80)**
SB2-A-2	SB2	3.71 mmol	333K, Acetone/water (20:80)
SB2-B-1	SB2	5.57 mmol	333K, Acetone/water (20:80)
SB2-B-2	SB2	5.57 mmol	333K, Acetone/water (20:80)
SB2-C-1	SB2	7.41 mmol	333K, Acetone/water (20:80)
SB2-C-2	SB2	7.41 mmol	333K, Acetone/water (20:80)
SB2-D-1	SB2	11.12 mmol	333K, Acetone/water (20:80)
SB2-D-2	SB2	11.12 mmol	333K, Acetone/water (20:80)

Column	Monomer	Monomer amount	EVO-Condition
		4 mm-Column, 3 g of PS-DVB polymer	
SB2-E-1	SB2	14.82 mmol	333K, Acetone/water (20:80)
SB2-E-2	SB2	14.82 mmol	333K, Acetone/water (20:80)
SB3-A-1	**SB3**	**1.26 mmol**	**333K, Acetone/water (20:80)**
SB3-A-2	SB3	1.26 mmol	333K, Acetone/water (20:80)
SB3-B-1	SB3	4.98 mmol	333K, Acetone/water (20:80)
SB3-B-2	SB3	4.98 mmol	333K, Acetone/water (20:80)
SB3-C-1	SB3	10.92 mmol	333K, Acetone/water (20:80)
SB3-C-2	SB3	10.92 mmol	333K, Acetone/water (20:80)
SB3-D-1	SB3	16.38 mmol	333K, Acetone/water (20:80)
SB3-D-2	SB3	16.38 mmol	333K, Acetone/water (20:80)
SB4-A-1	**SB4**	**2.49 mmol**	**333K, Acetone/water (20:80)**
SB4-A-2	SB4	2.49 mmol	333K, Acetone/water (20:80)
SB4-B-1	SB4	5.34 mmol	333K, Acetone/water (20:80)
SB4-B-2	SB4	5.34 mmol	333K, Acetone/water (20:80)
SB4-C-1	SB4	7.50 mmol	333K, Acetone/water (20:80)
SB4-C-2	SB4	7.50 mmol	333K, Acetone/water (20:80)
SB4-D-1	SB4	9.96 mmol	333K, Acetone/water (20:80)
SB4-D-1	SB4	9.96 mmol	333K, Acetone/water (20:80)

4.4 Determination of exchange capacities

After the zwitterionic monomers were successfully synthesized and characterized, the zwitterionic stationary phases could be prepared on PS/DVB-polymers using the grafting technique called EVO. The control adjustment of exchanger-capacities is of huge attraction

for the investigation of spacer length influence on zwitterionic stationary phases. Dynamic methods for the determination of exchange capacities in chromatography cannot be applied to sulfobetaine materials as they show intra- and intermolecular saturation of their charges [10]. Therefore, it is not possible to load and flush the materials with a distinct cation or anion without a partial elution of it. For this reason, the capacity of the zwitterionic exchangers are determined by a combination of powder x-ray fluorescence and elemental analysis. The shortcomings of these methods are the inability to provide the effective capacity of the exchangers like dynamic working methods. The information will be the total capacity of the exchanger. In other words, non-dynamic methods (x-ray fluorescence and elemental analysis) that provide "bulk capacity" which means they exhibit the all quantities of functional groups of the whole exchanger, whilst dynamical methods will offer "effective capacity", it will represent only the quantity of functional groups available for solutes. Table 4.3 shows the averaged capacities of the four sulfobetaine exchangers calculated from the sulfur content from XRF and the sulfur and nitrogen contents from elemental analysis.

Table 4.3: Capacities of the prepared zwitterionic exchangers with varying amount of zwitterionic monomer.

Chain length/methylene groups		SB_n-A	SB_n-B	SB_n-C	SB_n-D
Capacity / µeq g^{-1}	**n=1**	296	406	432	669
Capacity / µeq g^{-1}	**n=2**	300	585	590	790
Capacity / µeq g^{-1}	**n=3**	212	230	264	281
Capacity / µeq g^{-1}	**n=4**	250	265	284	298

Figure 4.8 shows the correlation between the sulfur and nitrogen contents (via elemental analysis) of zwitterionic exchangers. X-ray fluorescence and elemental analysis methods showed a good correlation (Figure 4.9) with a linear relationship over a wide range of capacities.

Notwithstanding the increase of the concentration of zwitterionic monomers leading to increased exchange capacity, but the capacity range of SB3 and SB4 exchangers is lower than the capacity range of SB1 and SB2 exchangers due to the steric hindrance of methylene groups and the graft polymerization procedure, as illustrated in Figure 4.9.

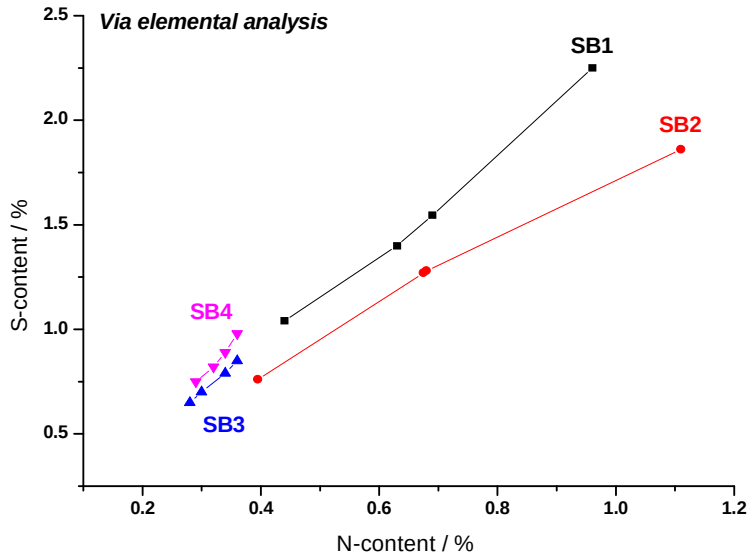


Figure 4.8: Correlation between the sulfur and nitrogen amounts via combustion elemental analysis.

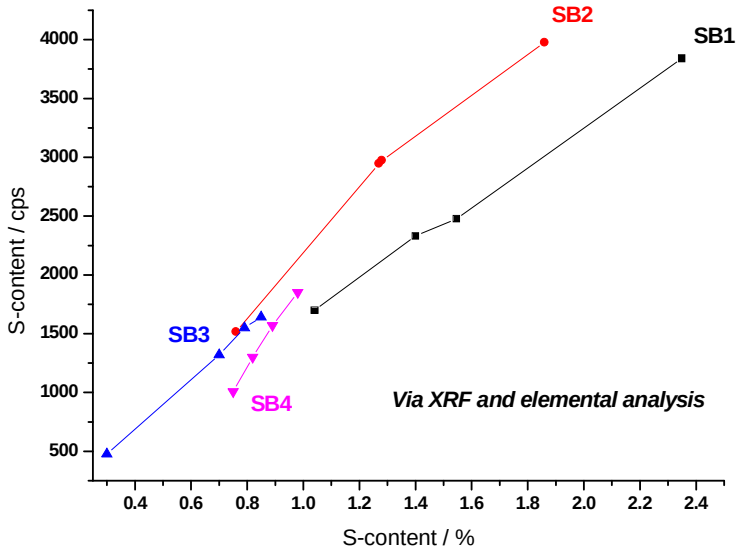


Figure 4.9: Correlation between the sulfur amount via X-Ray fluorescence and elemental analysis.

4.5 Chromatographic characterization under ZIC conditions

4.5.1 Separation of inorganic anions using acetate eluents

The chromatograms of the separations of five inorganic anions using sodium acetate eluents as characterization of the charge distance depend on properties in the ZIC separation mode using acetate buffers as eluents with UV detection are shown in Figure 4.10. The inorganic anions used as the targeted ions in this study were bromide, iodide, thiocyanate, nitrite and nitrate. The separation of anions was carried out with almost convergent capacities of the four-sulfobetaine exchangers. The exchanger with one-methylene groups (sulfobetaine SB1) shows the lowest retention times for all analytes and only 4 signals with a coelution of nitrite and bromide in the first signal, followed by nitrate, iodide and thiocyanate.

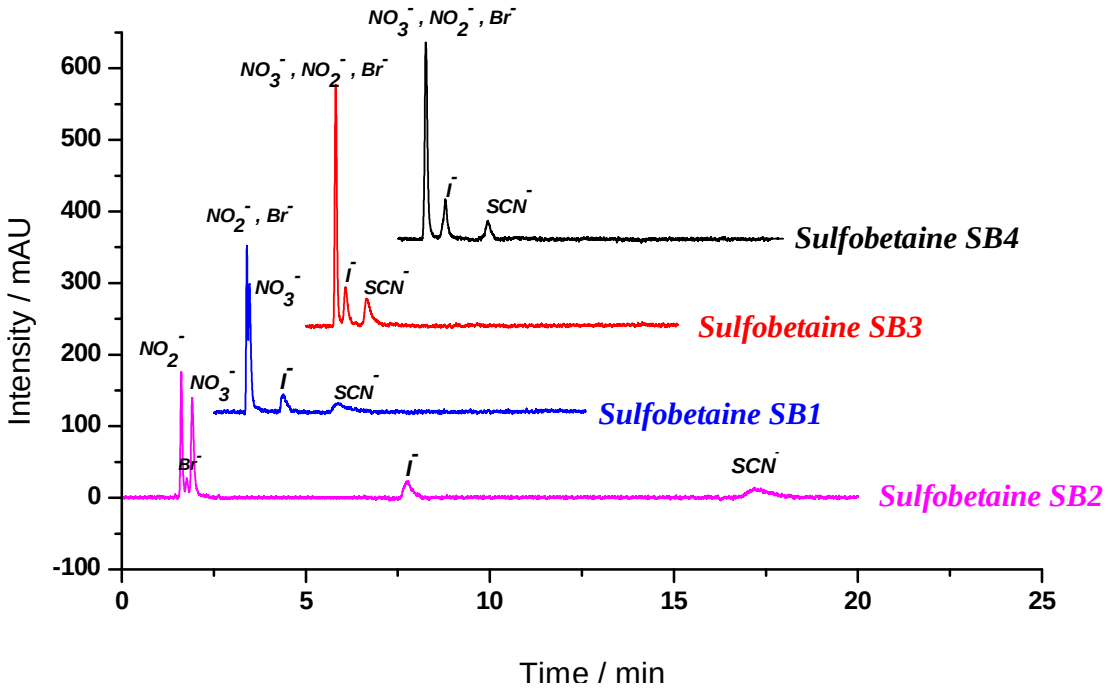


Figure 4.10: Separation of an anions mixture consisting of $NaNO_2$, NaBr, $NaNO_3$, NaI and NaSCN, eluent: 20 mM sodium acetate, pH 5.5, UV detection at 210 nm, flow rate 1.0 ml/min, temperature: 318 K.

For three (SB3) and four (SB4) methylene groups between the charges there are only three signals observable because of a coelution of nitrite, nitrate and bromide in the first signal, followed by iodide and thiocyanate. Interestingly, the exchanger with two (SB2) methylene groups between the charges separates all analytes and exhibits the highest retention for anions. The reason may be due to geometrical alignment of sulfobetaine groups. The study of the influence of pH and eluent concentration on anion selectivity should give a clue about the properties of the individual stationary phases and thus about the separation mechanism.

4.5.1.1 The influence of eluent concentration on retention of anions

The effect of ionic strength (10-40 mM) of the eluent on the separation of the anions was investigated using acetate buffers as eluents at pH 5.5 and temperature 318 K. The behavior of SB1, SB3 and SB4 exchangers is according to the suggested mechanism in ZIC [8, 12], which proposes an increasing *k'* with increasing ionic strength. They used zwitterionic surfactants (Zwittergent-3-14) adsorbed on an ODS material and suggested that a binary electrical double layer is created by the retention of the eluent anions and cations on positively and/or negatively charged functional groups of the zwitterionic surfactant forming an anion-EDL and cation-EDL. In other words, the increasing establishment of a binary electrical double layer increases k' with increasing ionic strength of the eluent. In Figure 4.11 and Figure 4.12, the retention factors for the targeted anions initially increased with increasing eluent concentration for all exchangers except the SB2-type. In a previous study [10], the sulfobetaine SB1 reached saturation of the EDL and retention factors on SB2 remained almost constant while varying eluent concentrations. This is contrary to what is observed in our study where the all the sulfobetaine columns prepared needed higher eluent concentrations to reach saturation of the EDL with exception of the SB2-exchanger. The reason for this difference in behavior of sulfobetaine exchangers toward varying ionic strength of the eluent is due to the difference in the capacity of the materials. The capacities used in the previous study ranged from 126-133 $\mu eq\ g^{-1}$ while this study utilizes capacities between 212 and 790-$\mu eq\ g^{-1}$. Increasing the capacity means increasing the amount of functional groups on the surface of the stationary phase, which leads in this case to an increased retention of the anions.

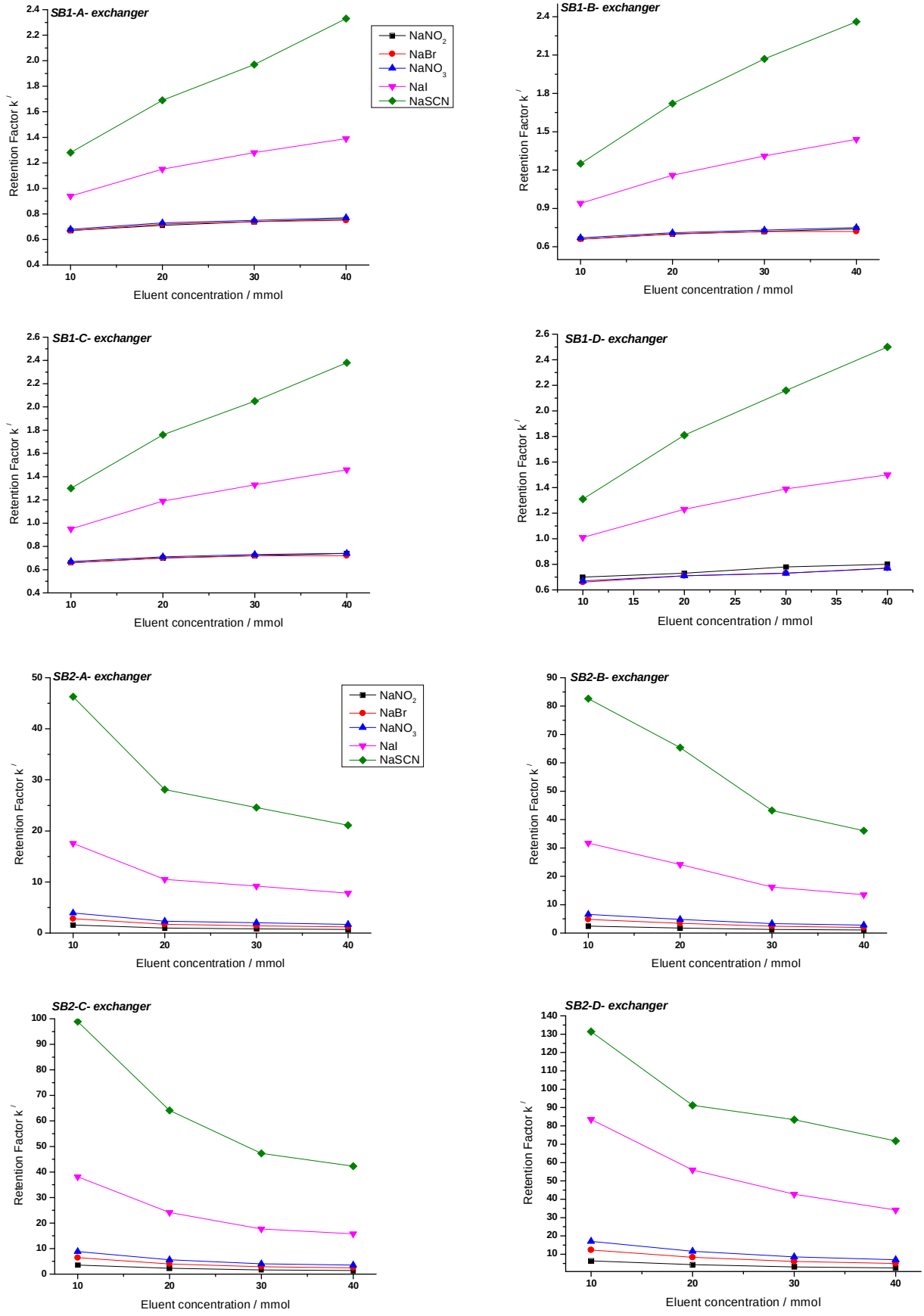


Figure 4.11: Variation of eluent concentration. Eluent: sodium acetate (pH 5.5), flow rate: 1.0 ml/min, temperature: 318 K, analyte concentration: 20 mg/kg.

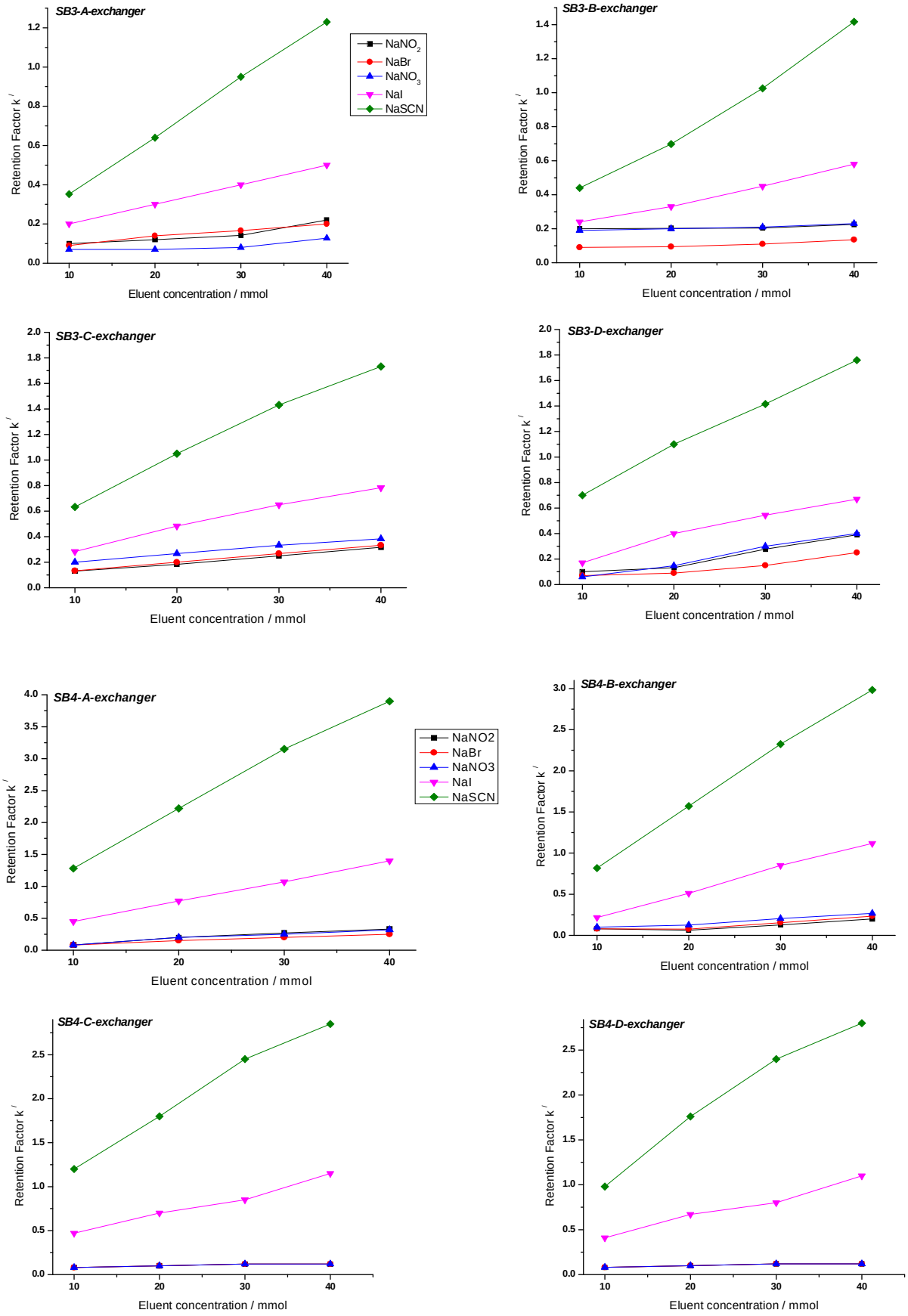


Figure 4.12: Variation of eluent concentration. Eluent: sodium acetate (pH 5.5), flow rate: 1.0 ml/min, temperature: 318 K, analyte concentration: 20 mg/kg.

The SB2 material shows a different mechanism in comparison to other zwitterionic exchangers. For the SB2 exchangers, it was found that the retention factor for anions decreased with the increase of the concentration of the eluent in the mobile phase. The reason might be that the SB2 distance between the charges do not allow intramolecular saturation of the charges [10]. Increasing concentration of the eluent leads to significantly weaker interactions [11]. The geometrical alignment seems to be important for this behavior and it is independent of exchange capacity (Figure 4.11, Figure 4.12). The slope of the plot of log *k'* versus log eluent concentration looks similar to the plots measured for standard anion exchange columns [43]. The relation between the retention factor *k'* for the solute ion and eluent concentration in the mobile phase, $E_m^{z_e}$, is given by Equation 4.1 [43]:

$$\log k' = C - \frac{z_s}{z_e} \log E_m^{z_e} \tag{4.1}$$

Here, C is the constant; z_e the charge eluent ion and z_s the charge solute ion. Both, the retention factor and eluent concentration values were logarithmically plotted and generally yield straight lines with a negative slope equal to ($-z_s/z_e$). Therefore, the behavior of SB2 can be explained as an almost pure anion exchange mechanism (Table 4.4).

Table 4.4: The slope values for log k' vs. log eluent concentration dependencies for SB2 columns in a different capacity.

Anion	NO_2^-	NO_3^-	Br^-	I^-	SCN^-
			SB2-A column		
R^2	0.9928	0.9920	0.9809	0.9771	0.9770
Slope	-0.5886	-0.6140	-0.6017	-0.5759	-0.5596
Intercept	0.7806	1.0533	1.1828	1.8051	2.2099
			SB2-B column		
R^2	0.9840	0.9793	0.9820	0.9601	0.9457
Slope	-0.6082	-0.6289	-0.6460	-0.6288	-0.6120
Intercept	1.0092	1.4649	1.3444	2.1521	2.5546

Anion	NO_2^-	NO_3^-	Br^-	I^-	SCN^-
			SB2-C column		
R^2	0.9967	0.9950	0.9971	0.9939	0.9940
Slope	-0.6694	-0.6673	-0.6673	-0.6527	-0.6305
Intercept	1.2202	1.6092	1.6092	2.2301	2.6238
			SB2-D column		
R^2	0.9928	0.9949	0.9947	0.9957	0.9871
Slope	-0.6597	-0.6389	-0.6540	-0.6390	-0.4338
Intercept	1.4732	1.8790	1.7526	2.5679	2.5435

4.5.1.2 The influence of eluent pH on the retention of anions

The effect of eluent pH was investigated over the pH interval 3 to 6 at different capacity levels using an eluent concentration of 20 mM sodium acetate. Nitrite behaves at pH values of 4 and lower oppositely to the other analytes. However, this is not an intrinsic property of the stationary phase but a property of nitrite (pKa of 3.29). All other analytes are salts of strong acids and, therefore, considered as completely dissociated. On SB1, SB3 and SB4 exchangers at pH 3, the retention factors are all low and increase with increasing pH (Figure 4.13, Figure 4.14). The reason for the increase in k' can only be seen in the increase in acetate and hydroxide concentrations or the decrease in hydron concentration [10]. It was found that the retention times for the targeted ions initially increased with the increase of pH in the mobile phase. Acetate and hydroxide concentrations rise with increasing pH. As long as saturation is not reached an increase in the hydroxide concentration outweighs the decrease in hydron concentration (SB3-exchangers in Figure 4.13).

In exchangers SB1, SB3 and SB4, we have observed that the retention factors for the analyte anions increase because their inner ammonium group is easier to reach. By increasing the concentration of hydroxide/acetate (increased pH), the shielding of the inner positive group should be reduced, which leads to a less negatively charged Donnan membrane. Consequently, this increases the retention of anions on the stationary phase.

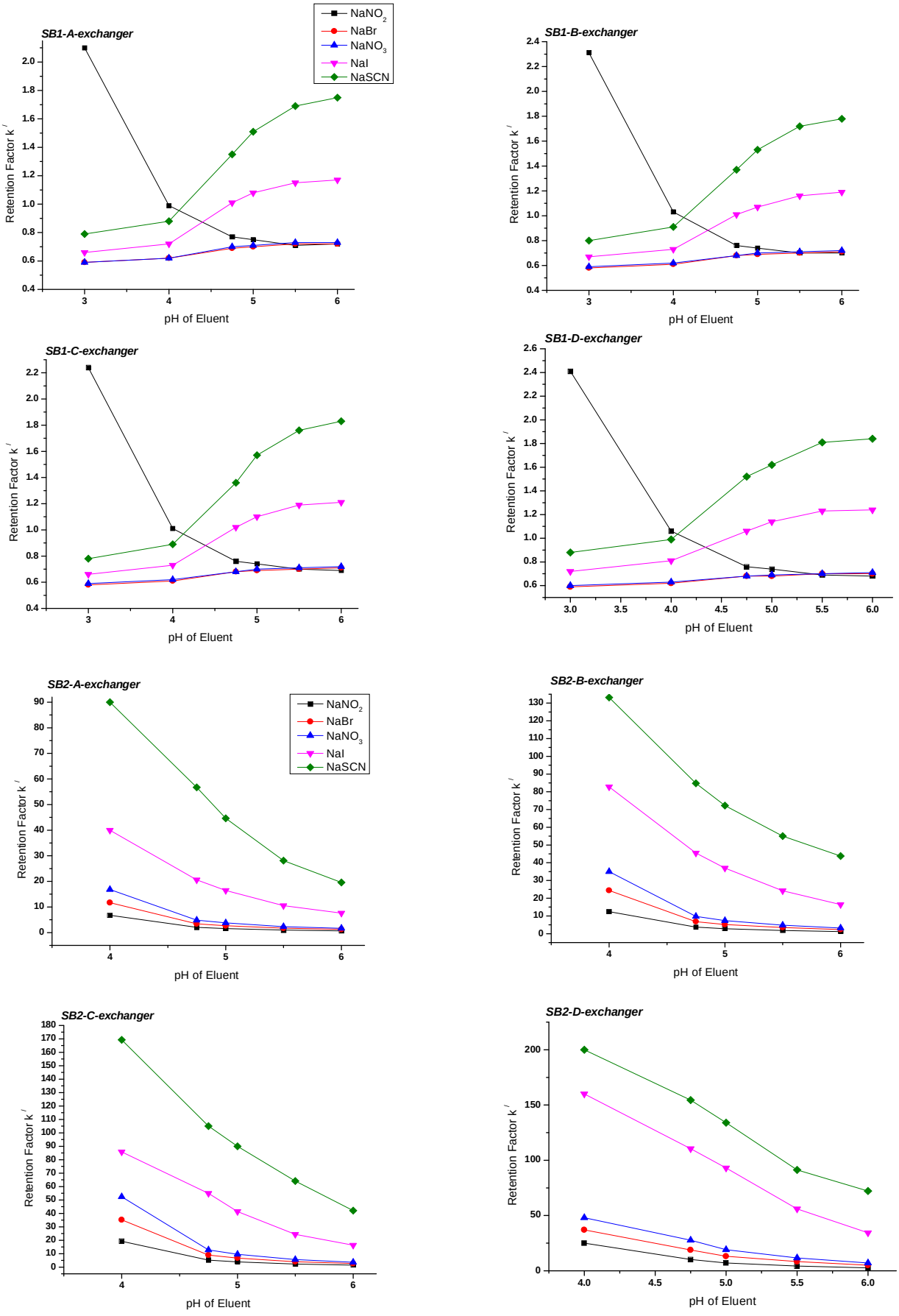


Figure 4.13: Variation of the pH of the eluent. Eluent: 20 mM sodium acetate, flow rate: 1.0 ml/min, temperature: 318 K, analyte concentration: 20 mg/kg.

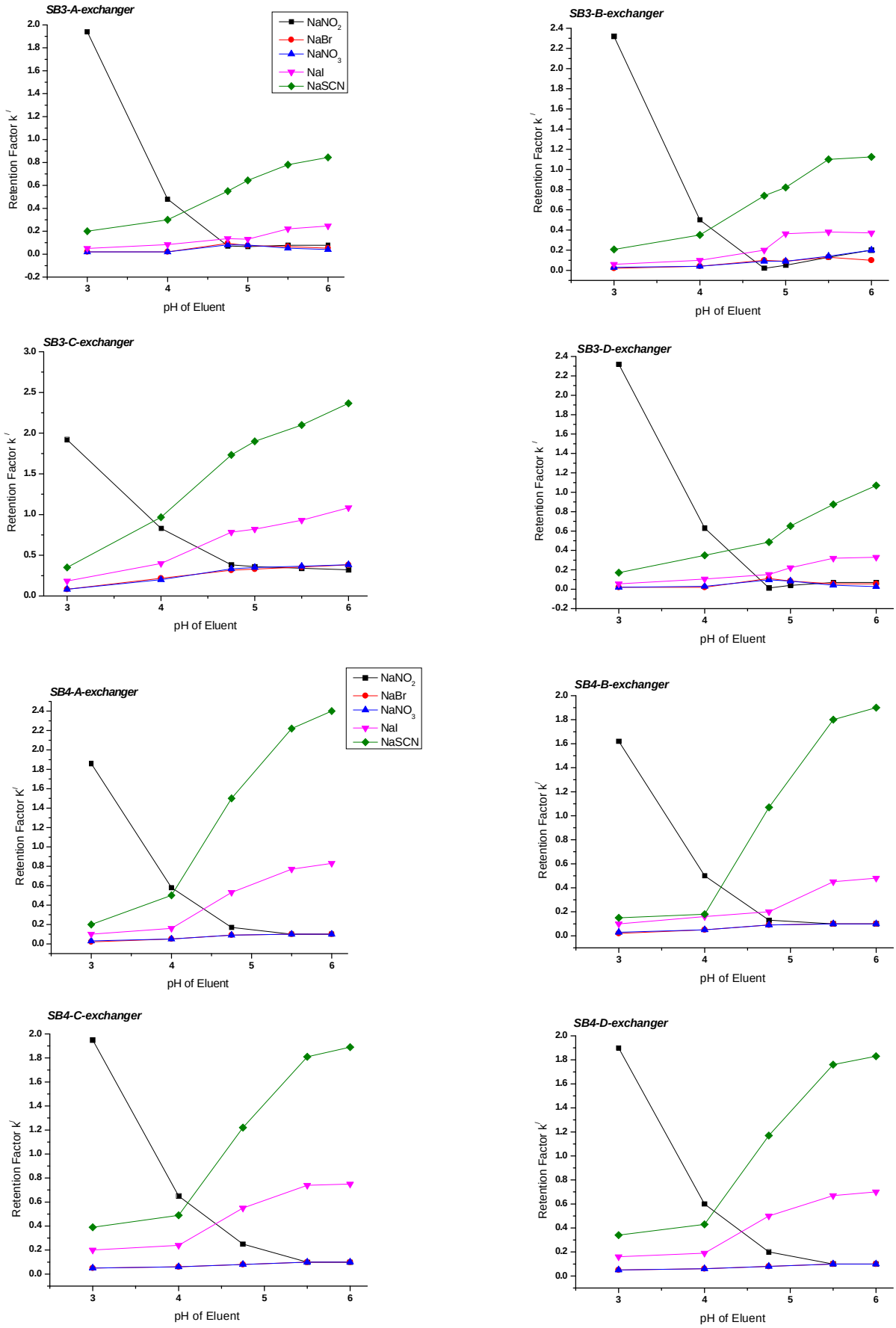


Figure 4.14: Variation of the pH of the eluent. Eluent: 20 mM sodium acetate, flow rate: 1.0 ml/min, temperature: 318 K, analyte concentration: 20 mg/kg.

The SB2-exchanger shows decrease in *k'* with an increasing pH (Figure 4.13). The reason for this effect must be the decrease in hydron concentration leading to a strong positive charged Donnan membrane [10, 14]. Strong shielding of H^+ was shown before [68]. The effects of eluent pH for the different capacities of SB1, SB2, SB3 and SB4 exchangers are shown in Figures 4.13 and 4.14.

4.5.1.3 The influence of exchange capacity on retention of anions

In general, increasing the concentration of the grafting solution (zwitterionic monomers) may lead to a promotion of the grafting of zwitterionic surfactants on PS-DVB copolymers. Therefore, the interaction between the anions and the larger number of functional groups on the stationary phase should increase. The following questions may arise: "What is the effect of the exchange capacity on selectivity and separation mechanism when varying the pH and eluent concentration?"and "Is there a correlation between exchange capacity and the spacer length between the charges and its effect on the retention of anions?"

After a complete investigation of the effect of pH in the range 3-6 and ionic strength of the eluent in the range (10-40 mM) on the retention of inorganic anions using four zwitterionic exchangers (SB1, SB2, SB3 and SB4) with various capacities for each column, we observed no change in the selectivity for the investigated inorganic anions and the order of selectivity remains NO_2^-< Br^-< NO_3^- < I^-< SCN^- for all sulfobetaine stationary phases independent of capacities. Moreover, it is worth mentioning that no change in the separation mechanism of anions has been observed except the SB2-exchanger. Retention factors for the four zwitterionic exchangers are plotted versus the capacity in Figure 4.15 at constant pH= 5.5 and eluent concentration (40 mM). Increasing capacity of the SB2 stationary phase results in increased retention factor for all anions (Figure 4.15). The distance between the charged groups could be sufficient to prevent inter- and intramolecular interactions.

The high values capacity of the SB2 column play a significant role in the separation mechanism of inorganic anions in comparison to the previous study [10] as noted previously. It is interesting to point out that increasing the capacities of the exchanger SB1 does not automatically lead to a significant change of the retention factor of anions (Figure 4.15).

The reason for this behavior can be attributed to the lowest polarity of SB1-exchanger in comparison to SB_n–exchangers, which mean SB1 has a rather inflexible geometry and, therefore, the charges get compensated intramolecularly [10].

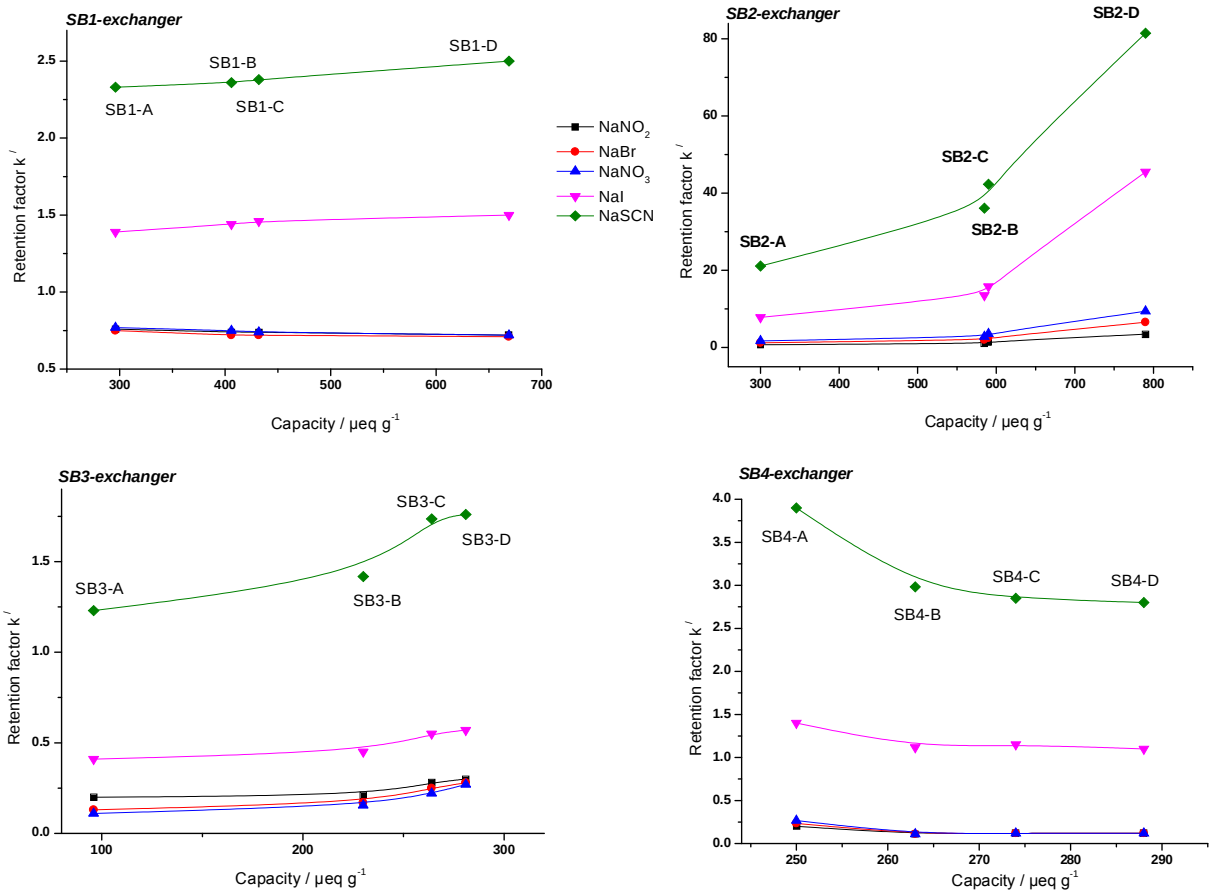


Figure 4.15: Influence of exchange capacity on retention of anions eluent: (40 mM sodium acetate, pH 5.5).

Increasing the capacity of the SB3 exchangers leads to a slight increase in the retention time of anions (Figure 4.15). However, the increase of the capacity of SB4 column led to a decrease in the retention factor of inorganic anions (Figure 4.15). A further increase in the grafting solution of SB_n-monomers may cause a reduced of free anion and cation sites available for analytes due to inter- and intramolecular effects. In other words, the chain length between the charges and exchange capacity of SBn-exchangers show an effect on the retention of inorganic anions.

4.6 Chromatographic characterization under ZIC-HILIC conditions

4.6.1 Separation of inorganic anions using acetate eluents under HILIC conditions

Anion separations under HILIC conditions are investigated. Inorganic ions cannot be counted to the classical HILIC analytes, although examples exist for the separation of anions and cations under ZIC-HILIC conditions [131-133]. The five anions are separated using sodium acetate eluents with a varying acetonitrile content. Retention of these analytes was observed for all capacities of the sulfobetaine-exchangers. The experiments were carried out to prove that organic modifiers (acetonitrile) could play a major role in the retention of inorganic anions. The chromatograms are shown in Figure 4.16.

Hydration enthalpies of inorganic ions should be an indication of the affinity to a water phase. Hydration enthalpies describe the amount of energy released when a mole of the ion dissolves in a large amount of water forming an infinite dilute aqueous phase [134]. The higher the energy (ΔH_{hyd}) released by ions, the higher the affinity of the ions should be to the aqueous phase and, therefore, the higher the retention under HILIC conditions should be. The hydration enthalpy values of the anions are summarized in Table 4.5.

Table 4.5: Hydration enthalpy of the investigated inorganic analytes. The hydration enthalpies refer only to the anions [134].

Inorganic anions	$NaNO_2$	NaBr	$NaNO_3$	NaI	NaSCN
Hydration enthalpy / kJ mol^{-1}	-380	-360	-314	-320	-310

Eluent conditions are changed methodically by starting with a variation of acetonitrile content, concentration and pH of eluent, in order to get a closer view into the mechanisms of

the separation of anions regarding the exchangers capacity and its impact on the retention time of anions.

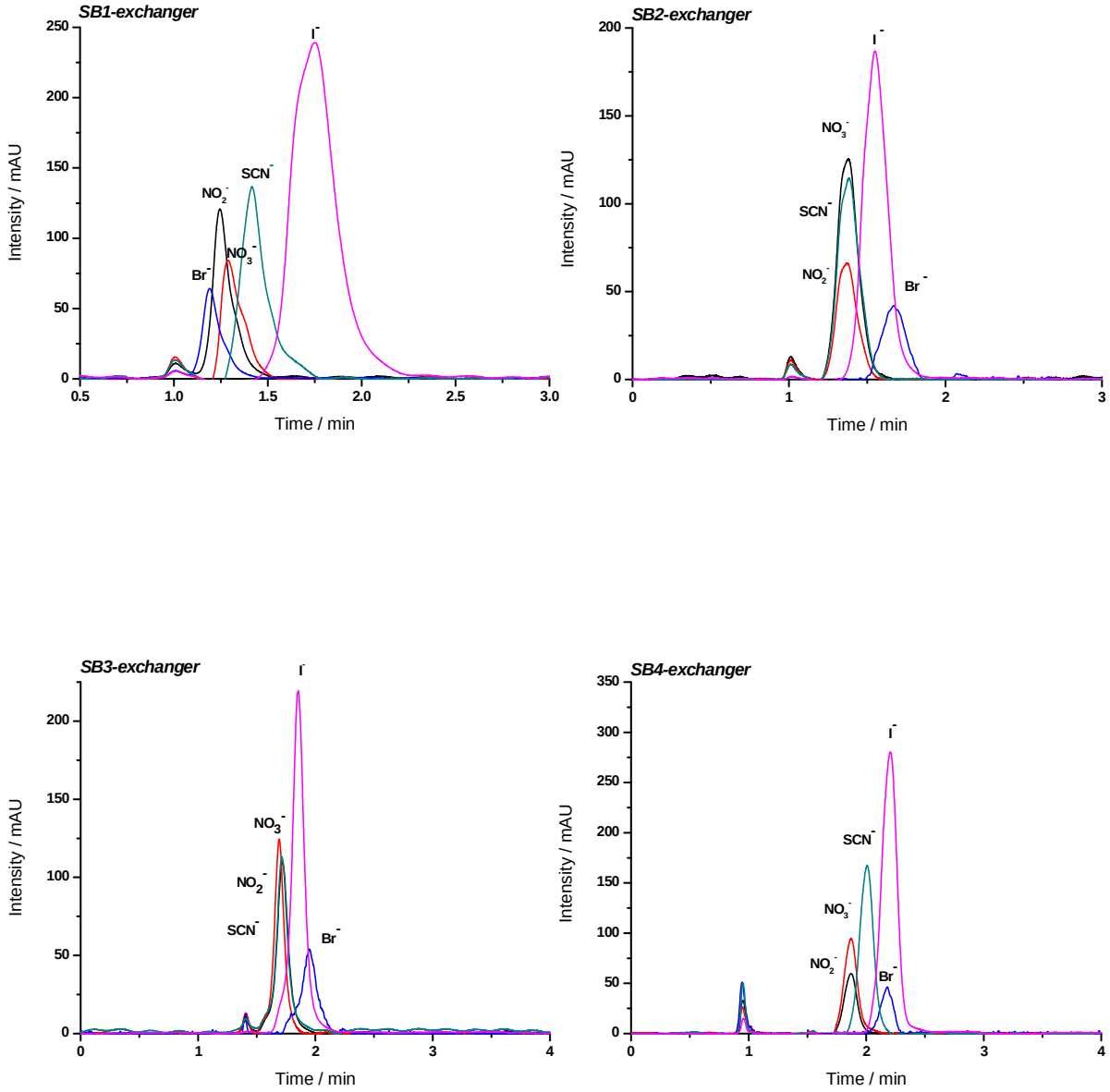


Figure 4.16: Separation of an anions mixture consisting of $NaNO_2$, NaBr, $NaNO_3$, NaI and NaSCN, eluent: (40 mM sodium acetate, pH 5.5, 80% acetonitrile), UV detection at 210 nm, flow rate 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

4.6.1.1 The influence of acetonitrile content on retention of anions

The ZIC-HILIC mechanism of anion separation is in contrast to the mentioned ZIC mechanism based on the distribution of the analytes between the water covered polar stationary phase and the less hydrophilic mobile phase (50-90% acetonitrile in water or a volatile buffer) via hydrophilic partitioning and electrostatic interactions. It is necessary that a certain amount of water is present in the mobile phase to ensure adequate hydration of the stationary phase particles [15, 17, 89].

In this study, the effect of acetonitrile content on retention was investigated by varying the percentage (20-80%) of acetontrile in the mobile phase while keeping sodium acetate concentration constant in the water phase at 40 mM (pH 5.5). It was observed for all SB-types that with increasing modifier content of 0% to 20% to 40% a decrease in retention factors for all anions takes place. At acetonitrile contents from 60% to 80% an increase in retention factors takes place with the exception of SB2-exchangers (Figure 4.17 and Figure 4.18). This means that SB1, SB3 and SB4 exchangers show HILIC behavior with at high acetonitrile contents in the mobile phase.

The selectivity of the anions followed the chaotropic Hofmeister series [14]; hence, the order of elution is NO_2^- > Br^-> NO_3^-> I^-> SCN^-. The chaotropic series of anions sorts according to their polarizability. The selectivity change under ZIC-HILIC conditions, particularly when we look at the plots for SB3-exchangers (Figure 4.18), means that bromide elutes last from the SB3-exchanger under HILIC conditions while it elutes first under ZIC conditions. The reason for this selectivity change must be a change in the separation mechanisms. Subsequently, the mechanism of separation could be based on a superposition of the chaotropic with the hydrophilic mechanism [62].

Because of the presence of a hydrophilic portioning mechanism due to the high affinity of inorganic anions to the aqueous phase, thus, solutes elution is facilitated by the polar component (water) of the mobile phase in HILIC-mode. However, this elution order is an effective tool in understanding the interactions of the separation.

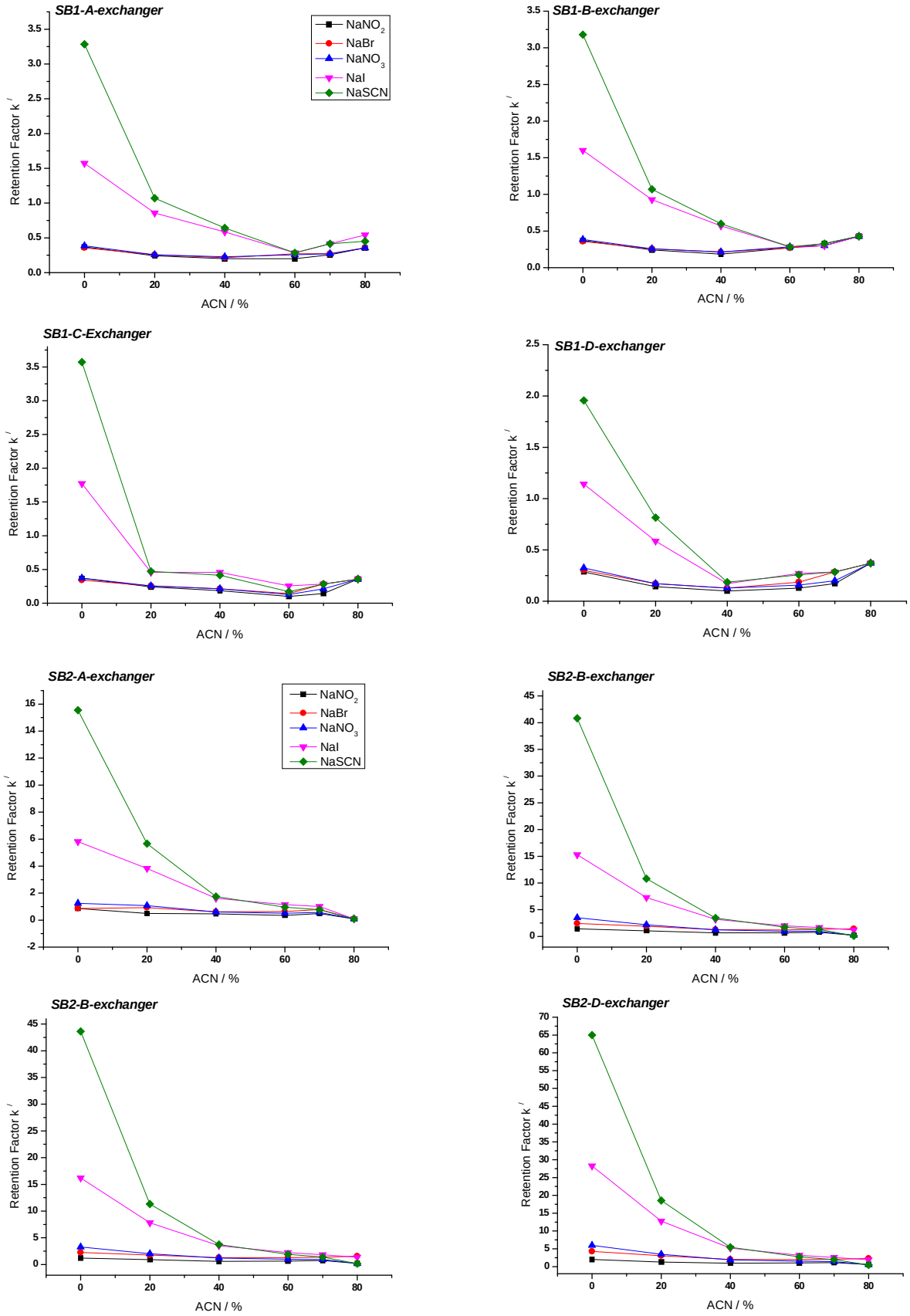


Figure 4.17: Separation of inorganic anions held with increasing modifier. Eluent: 40 mM sodium acetate, pH 5.5, UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

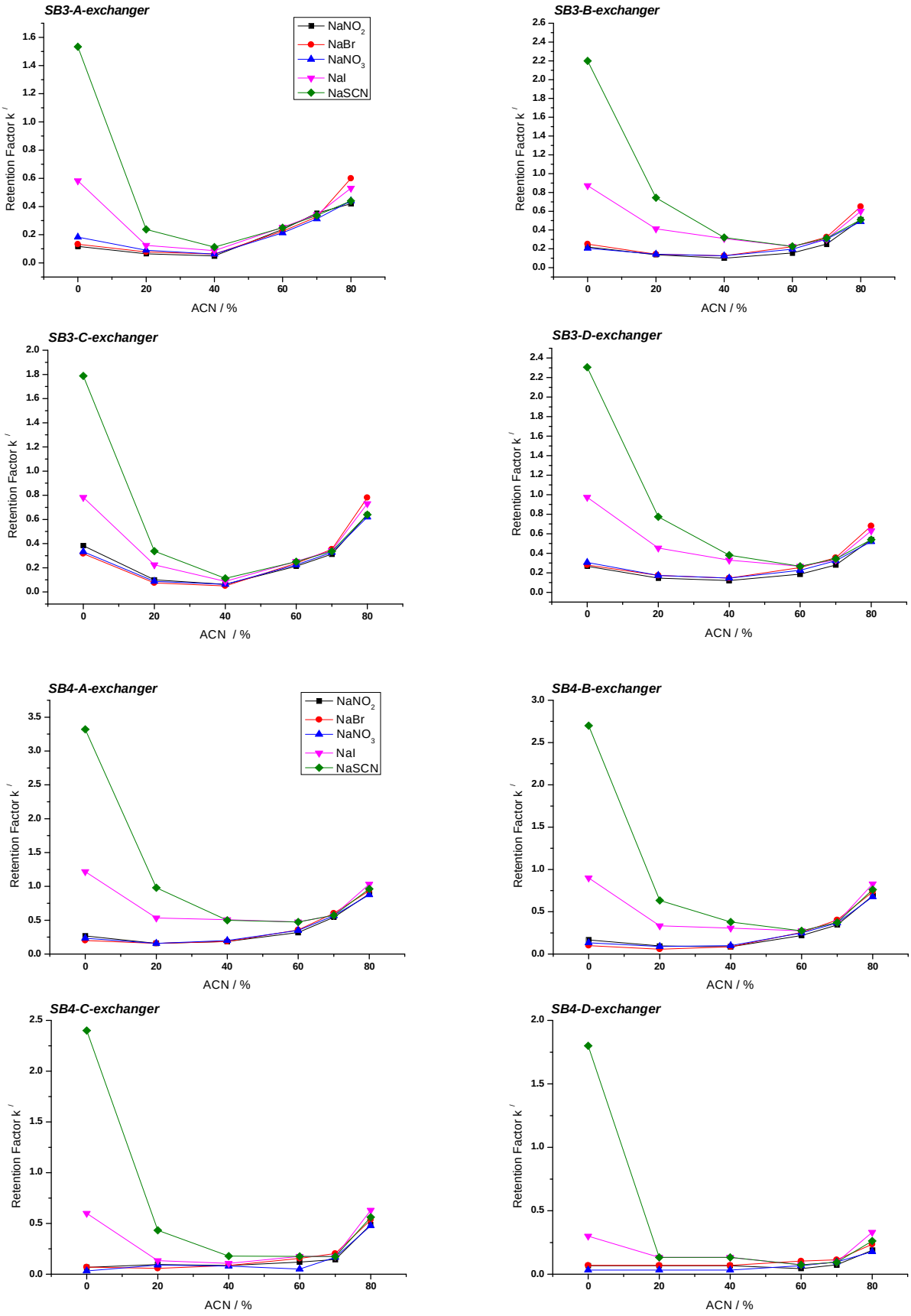


Figure 4.18: Separation of inorganic anions held with increasing modifier. Eluent: 40 mM sodium acetate, pH 5.5, UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

We know from Table 4.5 that the hydration enthalpy of bromide is significantly higher than that for other anions except for nitrite. In this case, for example, bromide and iodide have similar structures and they are, therefore, ideally suited for a comparison. The hydration enthalpy of bromide (-360 kJ mol^{-1}) is higher than that of iodide (-320 kJ $mole^{-1}$). Consequently, bromide has a much higher affinity to the aqueous phase. As a result, the elution of bromide and iodide will reverse on all investigated exchangers when switching between ZIC and ZIC-HILIC mode.

With nitrite having a hydration enthalpy -380 kJ $mole^{-1}$, one would expect a stronger retention than the one observed, behaving different due to superposition of the hydrophilic partitioning and the electrostatic interaction retention mechanism.

Interestingly, the SB2-exchangers show a reversed phase-like behavior of decreasing retention factors with increasing acetonitrile content in the mobile phase (Figure 4.17). Therefore, this behaves differently with regard to all other sulfobetaine-exchangers investigated in our work. We attribute this to the difference in the geometrical alignment.

The effect of the amount of organic modifier in the mobile phase on columns of different capacities has been studied. The exchanger SB1-B, SB3-C and SB4-A is the best to separate the anions and show the highest retention time for all anions at 80% acetonitrile. Increasing the exchanger capacity of these materials should not improve the ZIC-HILIC separation of inorganic anions performance significantly.

4.6.1.2 The influence of buffer strength on retention of anions

The next parameter in the eluent composition is a change in the buffer strength. When the buffer strength was increased from 20 to 80 mM sodium acetate (pH 5.5), the retention factor increased at constant acetonitrile fraction (80%). This effect has been observed for all sulfobetaine stationary phases (Figure 4.19, Figure 4.20).

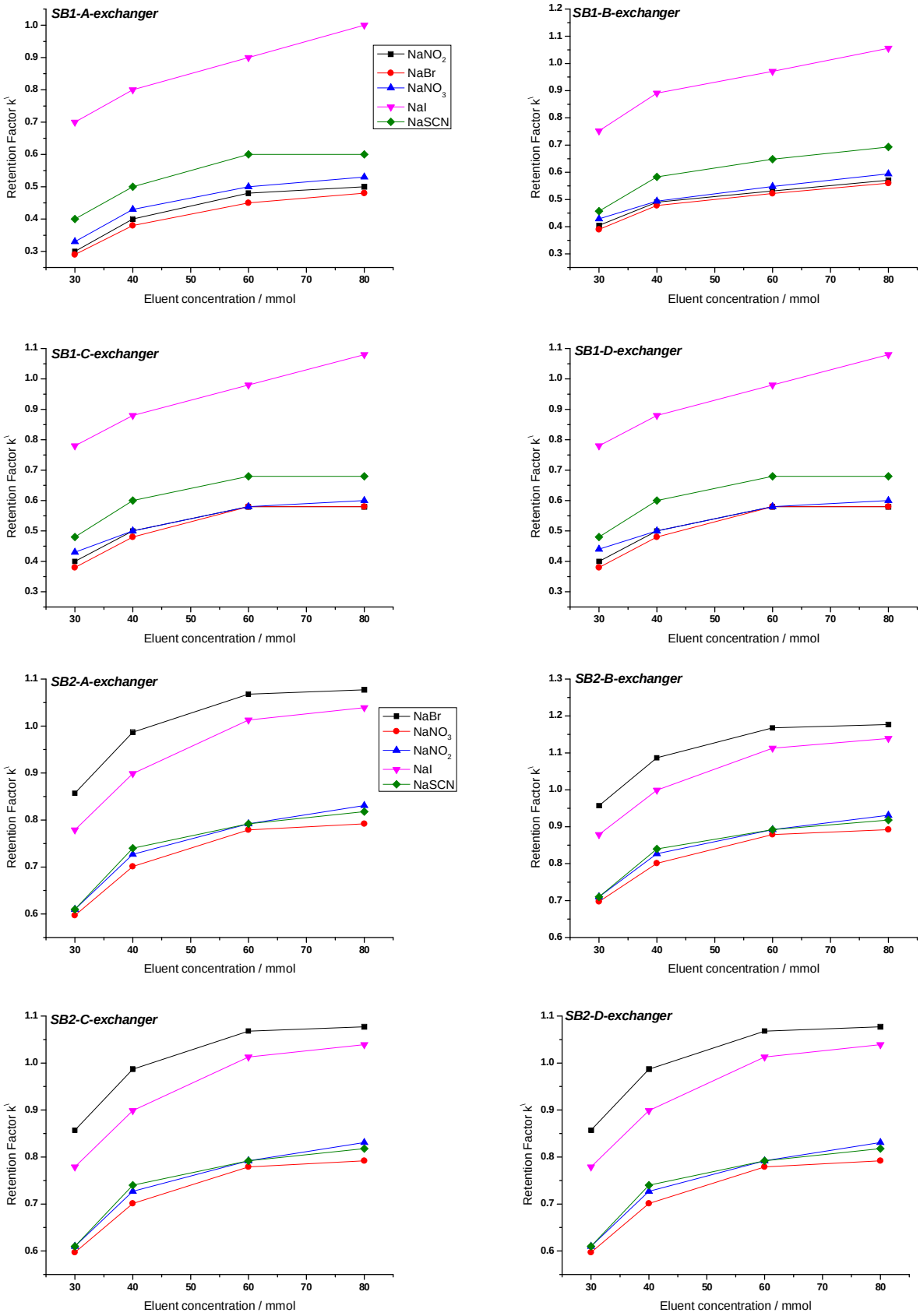


Figure 4.19: Variation of eluent ion concentration. Eluent: sodium acetate, pH 5.5, 80% acetonitrile. UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

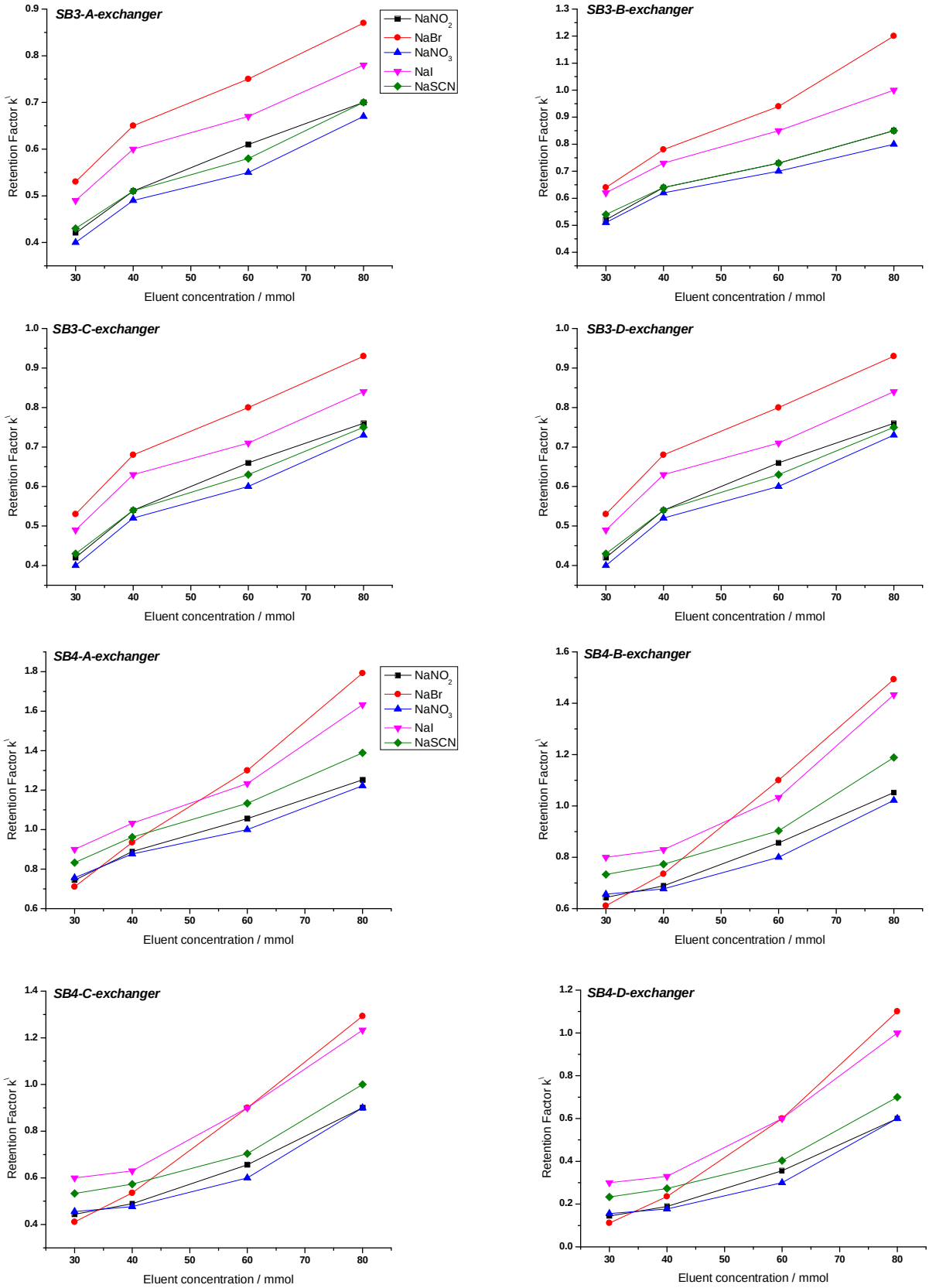


Figure 4.20: Variation of eluent ion concentration. Eluent: sodium acetate, pH 5.5, 80% acetonitrile. UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

Increasing the buffer strength should lead to a suppression of internal ion pairs and, therefore, promote the linearization of the functional groups of the stationary phase despite the presence of acetonitrile. This, in turn, improves the formation of a semi-immobilized aqueous adsorbed layer on the zwitterionic stationary phases, which lead to an increase of distribution capacity on the semi-stationary water phase [17]. In addition, increased acetate concentration enhances the enriched water layer on the polar stationary phase, which in turn increases the retention of anions [135].

Notable features that can be been seen in Figure 4.20 with regard to SB4-exchanger is a change in the selectivity of bromide. The order of selectivity for the SB4-stationary phase is $Br^- < NO_2^- < NO_3^- < SCN^- < I^-$ at low buffer strength (30 mM), whereas at high buffer strength (80 mM) the order of selectivity is $NO_3^- < NO_2^- < SCN^- < I^- < Br^-$. At a low buffer strength on SB4-exchangers, the formation semi-immobilized aqueous adsorbed layer on the zwitterionic stationary phase shall be limited and electrostatic interaction should be stronger [17, 62].

Eventually, under ZIC-HILIC condition, the retention mechanism of separation anions is according to hydrophilic partitioning and electrostatic mechanisms. Overall, it is worth mentioning that the variation of the eluent ionic strength in ZIC-HILIC mode revealed no significant difference between exchangers as compared to that which occurred when we studied this influence in ZIC-mode.

4.6.1.3 The influence of eluent pH on the retention of anions

In order to obtain a complete picture of anion separation under ZIC-HILIC condition, the eluent pH has to be varied. The influence of pH was evaluated over the range from pH 3 to pH 5.5 at constant buffer strength of 40 mM sodium acetate and constant acetonitrile fraction (80%). All exchangers showed comparable behavior to the observations under purely aqueous (ZIC-mode) conditions. At pH 3, the retention factors are low and increase when pH higher values (Figure 4.21, Figure 4.22), this behavior occurs on SB1, SB3 and SB4 exchangers. The reason for the increase in retention factors can only be seen in the increase in acetate/hydroxide concentrations or the decrease in hydrogen concentration.

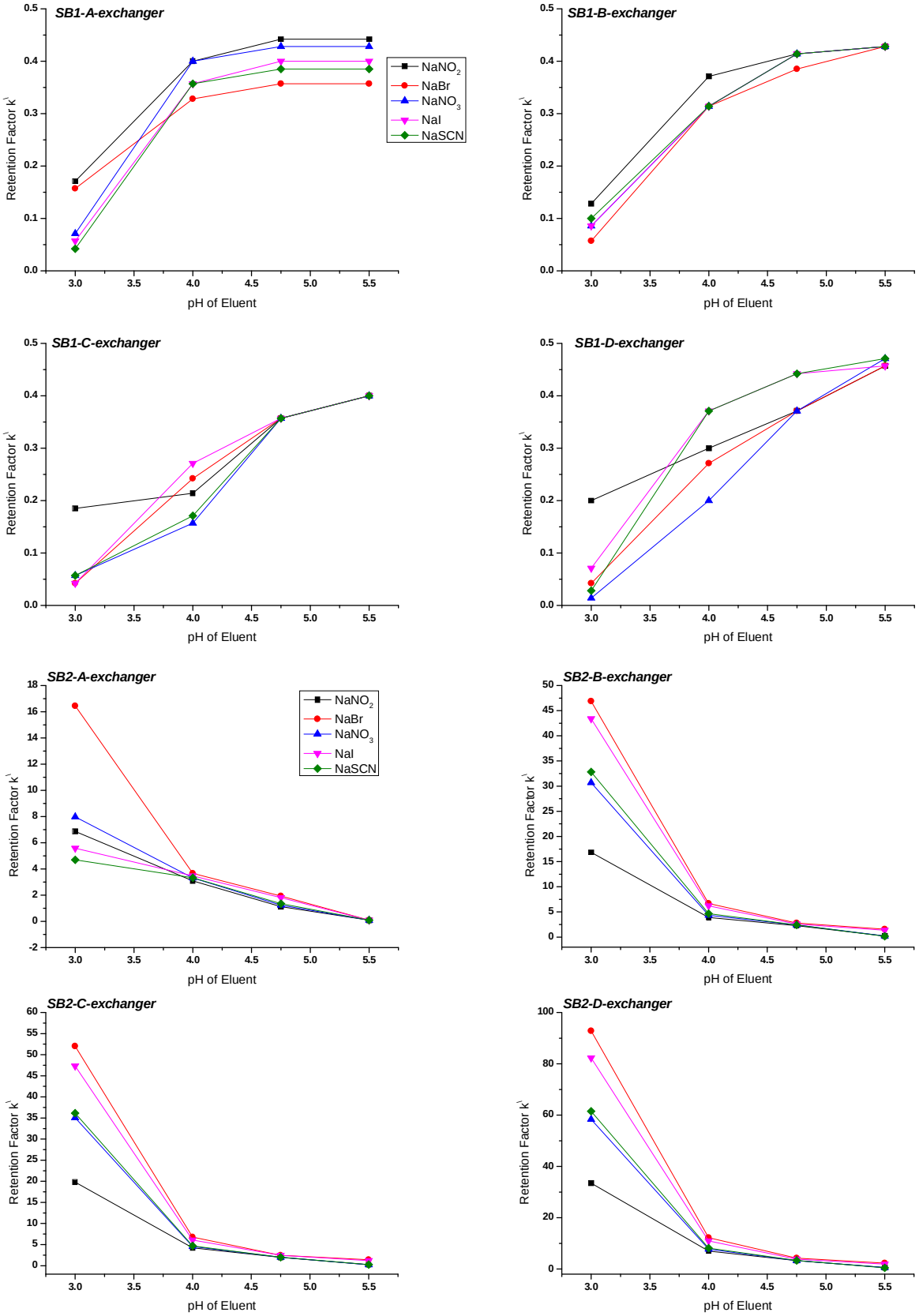


Figure 4.21: Variation of the pH of the eluent. Eluent: 20 mM sodium acetate, 80% acetonitrile. UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

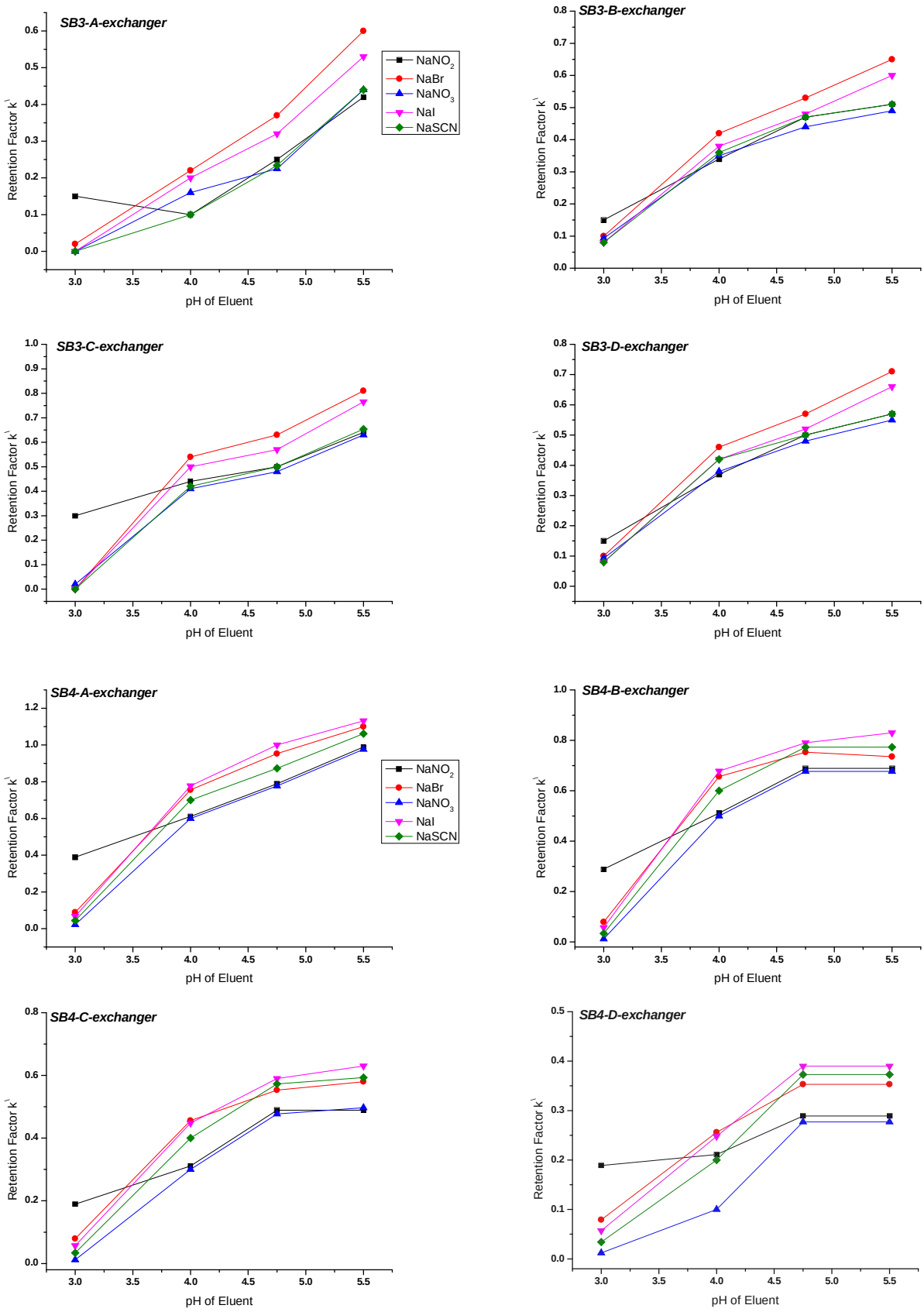


Figure 4.22: Variation of the pH of the eluent. Eluent: 20 mM sodium acetate, 80% acetonitrile. UV detection at 210 nm, flow rate: 0.75 ml/min, temperature: 318 K, analyte concentration: 10 mg/kg.

The formation of positive or negative charged Donnan membranes depending on the shielding of the functional groups using different anions and cations in the eluent [14] has different effects in accordance with the Hofmeister series [66]. This findings made on ZIC-mode [10] for the presence and the effect of Donnan membrane shall also apply to ZIC-HILIC mode. On the contrary, increasing pH leads to a decrease of the retention factors of the anions in the SB2 exchanger (Figure 4.21). The reason for this behavior must be due to the formation a strong positive charged Donnan membrane.

In sum, after the completion of all systematic variations of eluent conditions, we have to notice that the separation is based primarily on the hydrophilicity or the hydration enthalpy of the anions; electrostatic interactions play a minor role.

4.6.1.4 The influence of exchange capacity on retention of anions

We have already discussed the influence of the zwitterionic columns capacity on the separation of anions in ZIC mode (Paragraph 4.5.1.3). All exchangers show comparable behavior to the observations made for purely aqueous (ZIC-mode) conditions. After a complete image of the separation mechanism of inorganic anions under ZIC-HILIC mode, the upcoming question is the role played by the exchange capacity for the four-sulfobetaine materials in order to improve the ZIC-HILIC separation performance significantly.

It is noteworthy that no change in the selectivity order and separation mechanism of inorganic anions is observed when studying the influence of acetonitrile content (20-80%), pH (3-5.5) and buffer strength (30-80 mM) for different capacities for all types of sulfobetaine exchangers. It should be noted that the selectivity and separation mechanism of anions are changed due to differences in the spacer length between the charges and other factors that have already been noted previously. Retention factors for the four sulfobetaine exchangers are plotted versus the capacity in Figure 4.23 at constant 80% acetonitrile, pH= 5.5 and eluent concentration (80 Mm).

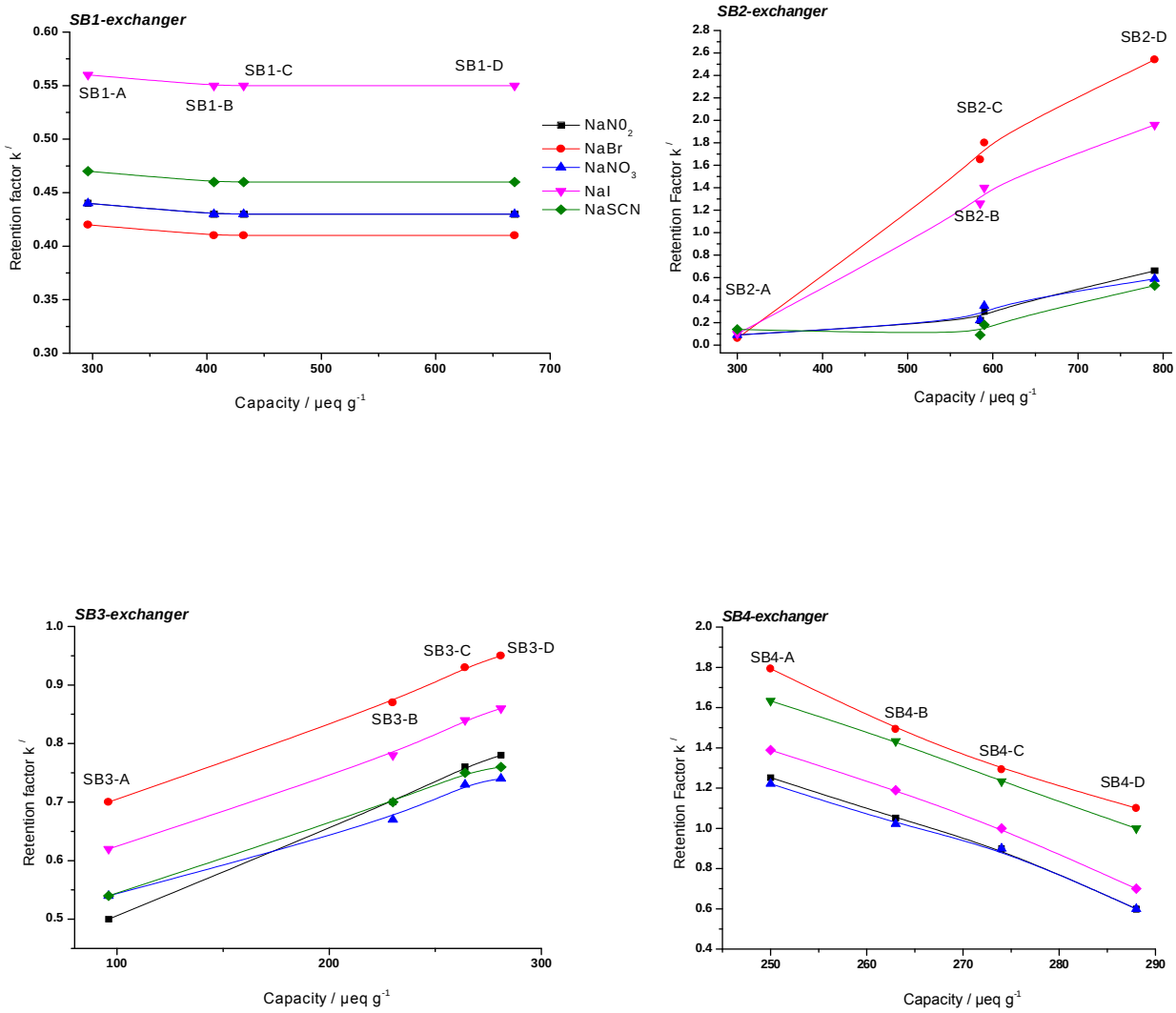


Figure 4.23: Influence of exchange capacity on retention of anions eluent: (80 mM sodium acetate pH 5.5, 80% acetonitrile)

As seen in Figure 4.23, there are different behaviors of zwitterionic columns. We note that increasing the capacity leads to an increase or decrease or no significant change in the retention of anions. The reason for this is due to the distance difference between positively and negatively charged sites combination in a single molecule attached the PS/DVB surface [10]. To answer the question that has been raised previously, we believe that the increase in capacity of columns that have been prepared did not improve the separation selectivity of anions.

4.6.2 Separation of pharmaceuticals using acetate eluents under HILIC conditions

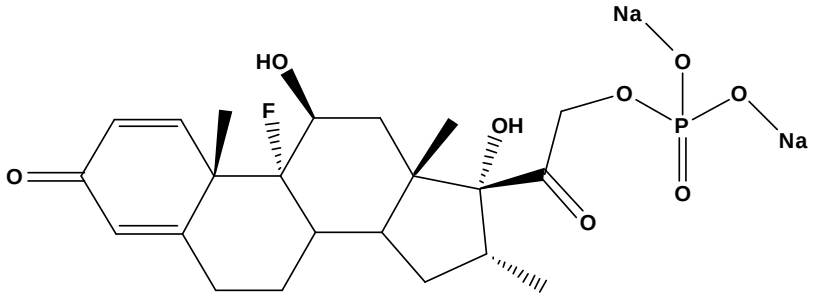
The study aimed the application of ZIC-HILIC using the new SB1-SB4 columns to eight relevant pharmaceutical compounds with chemical structures as shown in Figure 4.24. The pharmaceuticals were selected with respect to their polarity:

Polar pharmaceuticals:
- Deferoxamine mesylate (DFOM)
- Thiamine. HCl (B1)

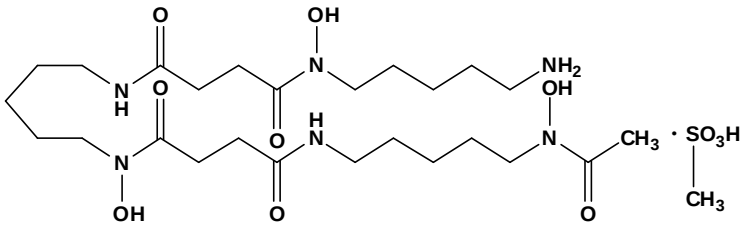
Non-polar:
- Diclofenac sodium (DCS)
- Cyclopentolate. HCl (CPH)
- Dexamethasone sodium phosphate (DMSP)
- Tetracaine. HCl (TCH)
- Pilocarpine. HCl (PCH)
- Chloramphenicol (CAP)

The pharmaceuticals were chosen as test probes for studying the hydrophilic interaction chromatographic properties when applying a sodium acetate eluent with varying acetonitrile content using the homologous row of sulfobetaine exchangers. A significant retention of all eight-model compounds can be achieved with a sodium acetate eluent at a concentration of 20-40 mM with 65-70% acetonitrile content and pH 4.75. Chromatograms are shown in Figure 4.25. The highest retention factors and, therefore, the best separations under these conditions were achieved using the sulfobetaine SB3 and SB4 exchangers.

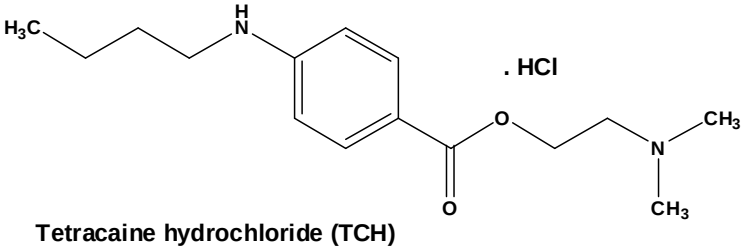
The test set should allow to recognize the influence of spacer length on the separation of compounds of different polarity. After investigation of the chromatograms for the model compound, the eluent composition is varied systematically by variation of acetonitrile content, buffer concentration and pH of the eluent to get a closer vision into the mechanisms of the separation of model compounds with respect to the spacer chain length.



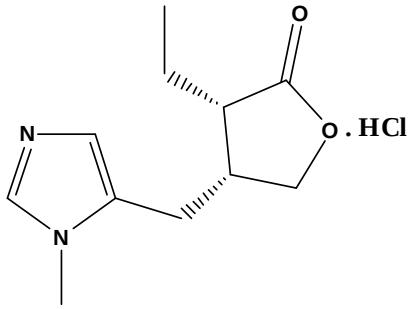
Dexamethasone Sodium Phosphate (DMSP)



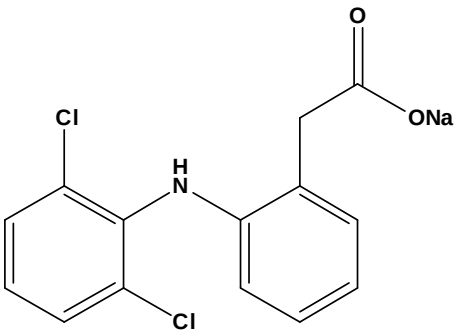
Deferoxamine mesylate (DFOM)



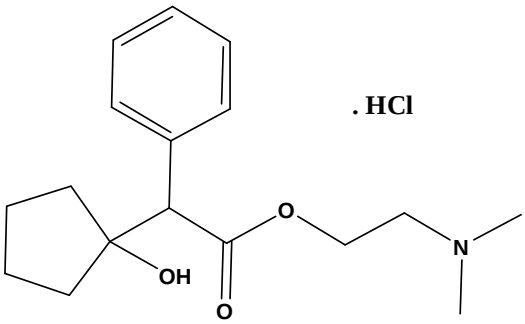
Tetracaine hydrochloride (TCH)



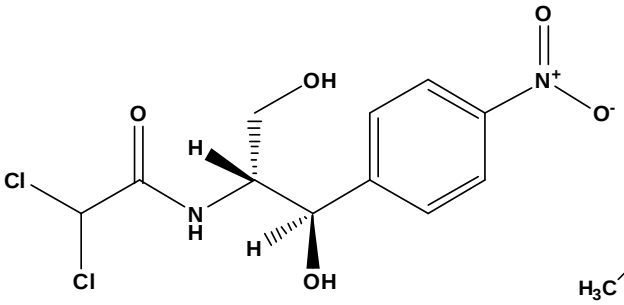
Pilocarpine hydrochloride (PCH)



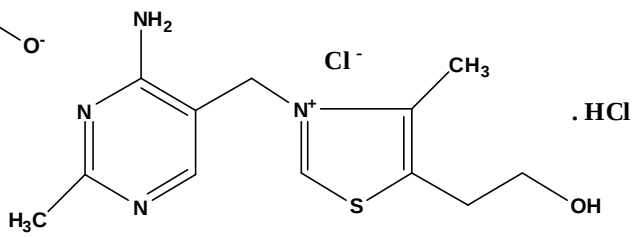
Diclofenac sodium (DCS)



Cyclopentolate hydrochloride (CPH)



Chloramphenicol (CPA)



Thiamine hydrochloride (B1)

Figure 4.24: Structural formulas of tested compounds investigated in the present study.

Table 4.6: Capacities of the zwitterionic exchangers used for the separation of pharmaceuticals under ZIC-HILIC conditions.

Chain length / methylene groups	1	2	3	4
Capacity / µeq g^{-1}	296	300	264	265

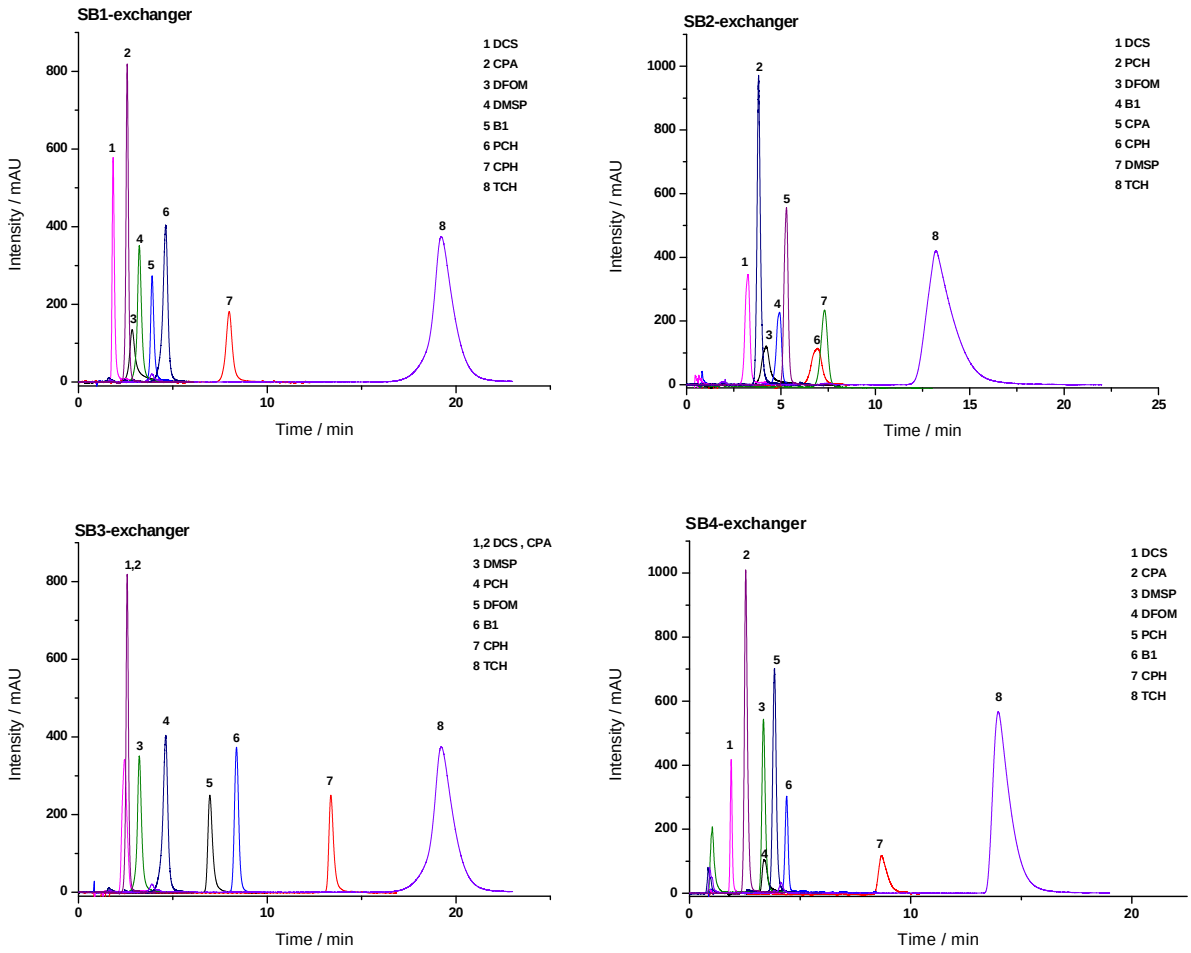


Figure 4.25: Chromatograms for all tested compounds separated on SB_n-columns. Mobile phase: acetonitrile/sodium acetate (70/30) containing 40 mM sodium acetate (pH=4.75) for SB1, SB4; acetonitrile/sodium acetate (65/35) containing 20 mM sodium acetate (pH=4.75) for SB2 and SB3. Flow rate: 0.75 ml/min. Column temperature 318 K. Analyte concentration: 50 mg/kg. UV detection: 215 nm for DFOM, PCH and CPH; 260 nm for B1; 274 nm for DCS, CAP; 255 nm for DMSP; 310 nm TCH.

4.6.2.1 Variation of acetonitrile content

The mobile phase used in HILIC resemble the solvents of reversed phase liquid chromatography (RP-LC) [15] but requires much higher organic content to ensure significant hydrophilic interaction [17]. The separation mechanism in HILIC is based on the distribution of polar solute between the water-enriched layer onto HILIC-column and the acetonitrile-rich mobile phase [17]. The fraction of the organic solvent in the mobile phase has the largest influence on the retention of polar and non-polar compounds. In the current work, the effect of acetonitrile content on retention was investigated by varying the volume fraction of acetonitrile in the mobile phase while keeping sodium acetate concentration constant at 40 mM (pH 4.75).

Both, DFOM and B1 show HILIC behavior with increasing retention factors with increasing acetonitrile content in the mobile phase (Figure 4.26). On the contrary, CPH, DCS, DMSP, CAP, PCH and TCH show RP behavior with decreasing retention factors with increasing acetonitrile content in the mobile phase (Figure 4.26). The reason for this difference in the behavior of the model compounds when varying acetontrile content in the mobile phase is due to the hydrophilicity of compounds, as it is clear from the log P_{ow} values of the compounds.

DFOM and B1 have a significantly increased hydrophilicity compared to CPH, DCS, DMSP, CAP, PCH and TCH. The log P_{ow} values confirm the strong secondary interaction of CPH, DCS, DMSP, CAP, PCH and TCH observed due to its low hydrophilicity. Some physical-chemical properties of these model compounds are summarized in Table 4.7.

Polar compounds such as DFOM and B1 increase the retention factors with increasing modifier contents while the non-polar compounds CPH, DCS, DMSP, CAP, PCH and TCH only show a stagnation of retention time. The plots in Figure 4.26 show the striking similarity in the behavior of sulfobetaine exchangers when studying the effect of acetonitrile content on the separation of the model compounds while we observe a significant difference in the behavior of compounds.

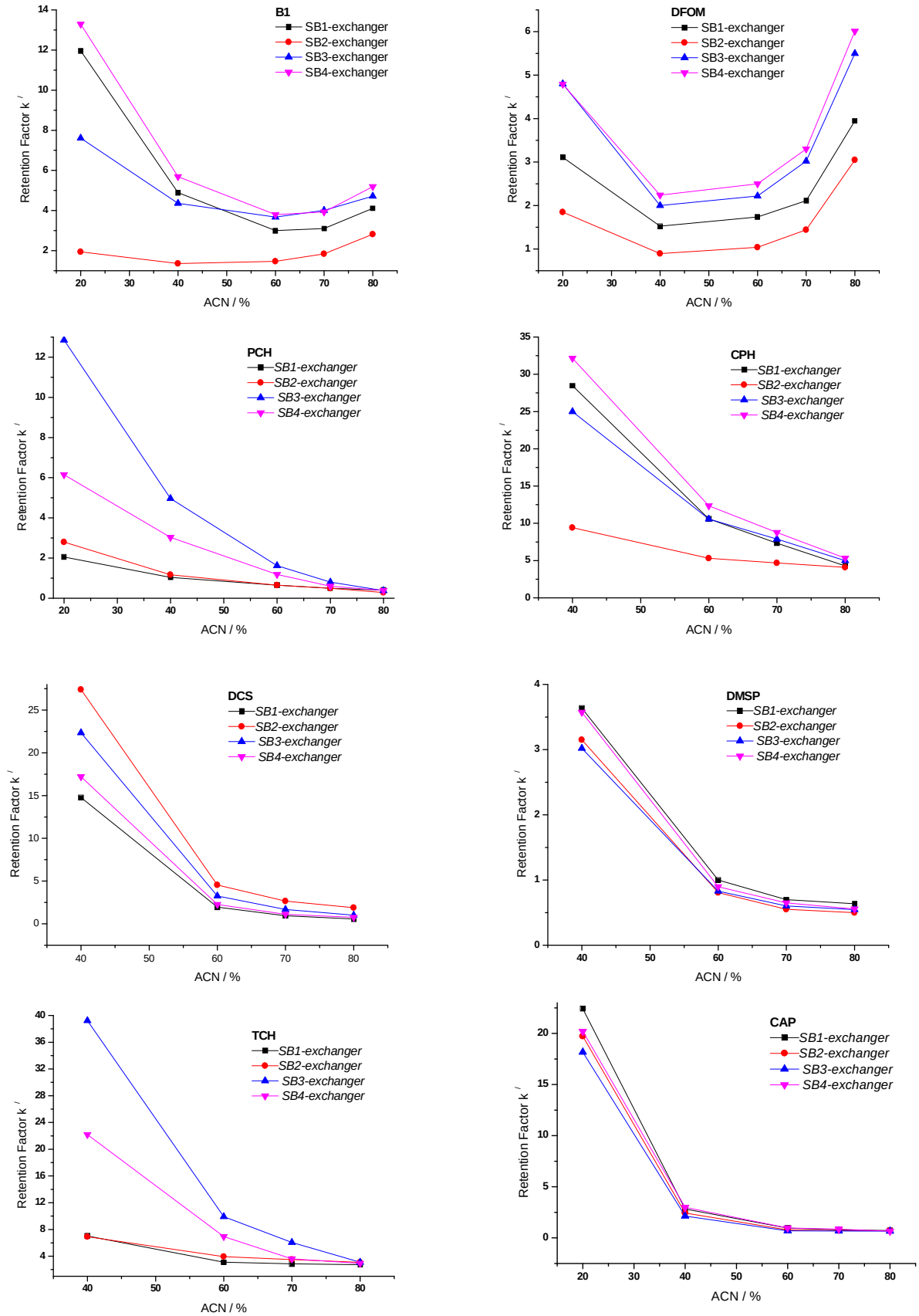


Figure 4.26: Separation of tested compounds with variation of the modifier. Eluent: 40 mM sodium acetate, 80% acetonitrile, pH 4.75; flow rate: 0.75 ml/min; temperature: 318 K; analyte concentration: 50 mg/kg.

Table 4.7 : Physical-chemical properties of the eight tested compounds [136, 137].

Compound	pI [137]	pK_a [136]	logP_{ow} [136]
1. Deferoxamine mesylate	7.88	7.9 ± 0.4 10.6 ± 0.4	-2.75 ± 0.76
2. Thiamine hydrochloride		3.8 ± 0.6 13.8 ± 0.5	- 2.5 ± 0.5
3. Pilocarpine hydrochloride		7 ± 0.4	-0.1 ± 0.33
4. Dexamethasone Sodium Phosphate	4.75	1.2 ± 0.4 6.1 ± 0.7 13.9 ± 0.9	0.65 ± 0.62
5. Tetracaine hydrochloride		8.2 ± 0.4	3.65 ± 0.54
6. Diclofenac sodium	0.96	4.4 ± 0.4	4.06 ± 0.41
7. Cyclopentolate hydrochloride	11.30	8.1 ± 0.4 14.2 ± 0.9	2.59 ± 0.34
8. Chloramphenicol	2.38	9.3 ± 1.2	1.02 0.37

4.6.2.2 Relationship between retention factor and mobile phase composition

The behavior of six investigated compounds (CPH, DCS, DMSP, CAP, PCH and TCH) shows reversed phase behavior due to their hydrophobicity. Hydrophobicity parameters have been calculated by linear correlation between the logarithm retention factor (*log k´*) of the investigated compounds and the fraction of acetonitrile (φ_{ACN}) in the mobile phase.

The empirical relationship using linear extrapolation to obtain hydrophobicity constants [138, 139] can be described by Equation 4.2:

$$\log k' = \log k'_w - S\varphi_{ACN} \quad (4.2)$$

Here, - *S* is the slope and *log k´w* the intercept of the regression curve. A good linear correlation R^2 was obtained between *log k´* and φ_{ACN} (Table 4.8). The *log k´w* value is a widely used chromatographic hydrophobicity parameter.

Interestingly, a significant correlation between *log k´w* and *log P* is only given for SB1, SB3 and SB4 columns (Figure 4.27). The SB2 column behaves differently. Remarkably, the correlation between *log k´w* and *log P* increases from SB1 over SB3 up to SB4. The SB4-exchanger exhibits the best linear relationship between *log k´w* and *log P* in comparison to all other SB_n-exchangers.

The *log P* values were calculated with the program ACD/logP, version 5.0.0184, using the ACD/I-Lab 2 (Table 4.7) [136]. *Klára et al.* [140] introduced a second hydrophobicty parameter, the hydrophobic index φ_0, which refers to the acetonitrile concentration in the mobile phase and which is required for *log k´* = 0; that is, the molar fraction of the compound is identical in the mobile and the stationary phases. This means that the higher the value of φ_0 the more hydrophobic the compound. The experimental values of the hydrophobic chromatographic index φ_0 were determined by using the intercept (*log k´w*) and slope (-*S*) [140].

Table 4.8 shows the results obtained for *log k´w*, *S,* φ_0 and R^2 for four sulfobetaine exchangers. Based on the hydrophobicity index φ_0 values, the sequence of compounds (CPH>TCH> DCS >CAP>) is similar for all sulfobetaine exchangers, except for DMSP and PCH. Regrettably, the correlation between φ_0 and *log P* of the investigated pharmaceuticals is unsatisfactory (Table 4.7).

Table 4.8: Regression results from Equation 4.2 for the tested compounds.

Comp./Column		CPH	DCS	DMSP	TCH	PCH	CPA
SB1-column	**log k'_w**	2.281	2.572	1.598	1.287	0.518	1.880
	S	-0.020	-0.036	-0.026	-0.011	-0.011	-0.029
	Φ_0	109.3	71.4	61	114.7	44.5	63.21
	R^2	0.9973	0.9905	0.9974	0.9934	0.9896	0.9826
SB2-column	**log k'_w**	1.326	2.604	1.589	1.229	0.709	1.732
	S	-0.009	-0.030	-0.027	-0.009	-0.014	-0.028
	Φ_0	144.8	86.6	57.6	124.9	49.5	61.31
	R^2	0.9866	0.9782	0.9975	0.9988	0.9943	0.9496
SB3-column	**log k'_w**	2.063	2.662	1.535	2.656	2.166	1.710
	S	-0.017	-0.033	-0.026	-0.027	-0.029	-0.032
	Φ_0	121.3	79.2	57.8	98.2	59.18	66.99
	R^2	0.9940	0.9860	0.9972	0.9960	0.9553	0.9994
SB4-column	**log k'_w**	2.241	2.742	1.626	2.33	1.239	1.816
	S	-0.019	-0.038	-0.027	-0.024	-0.020	-0.029
	Φ_0	118.5	71	59.6	94.1	61.30	61.9
	R^2	0.9925	0.9937	0.9956	0.9940	0.9884	0.9804

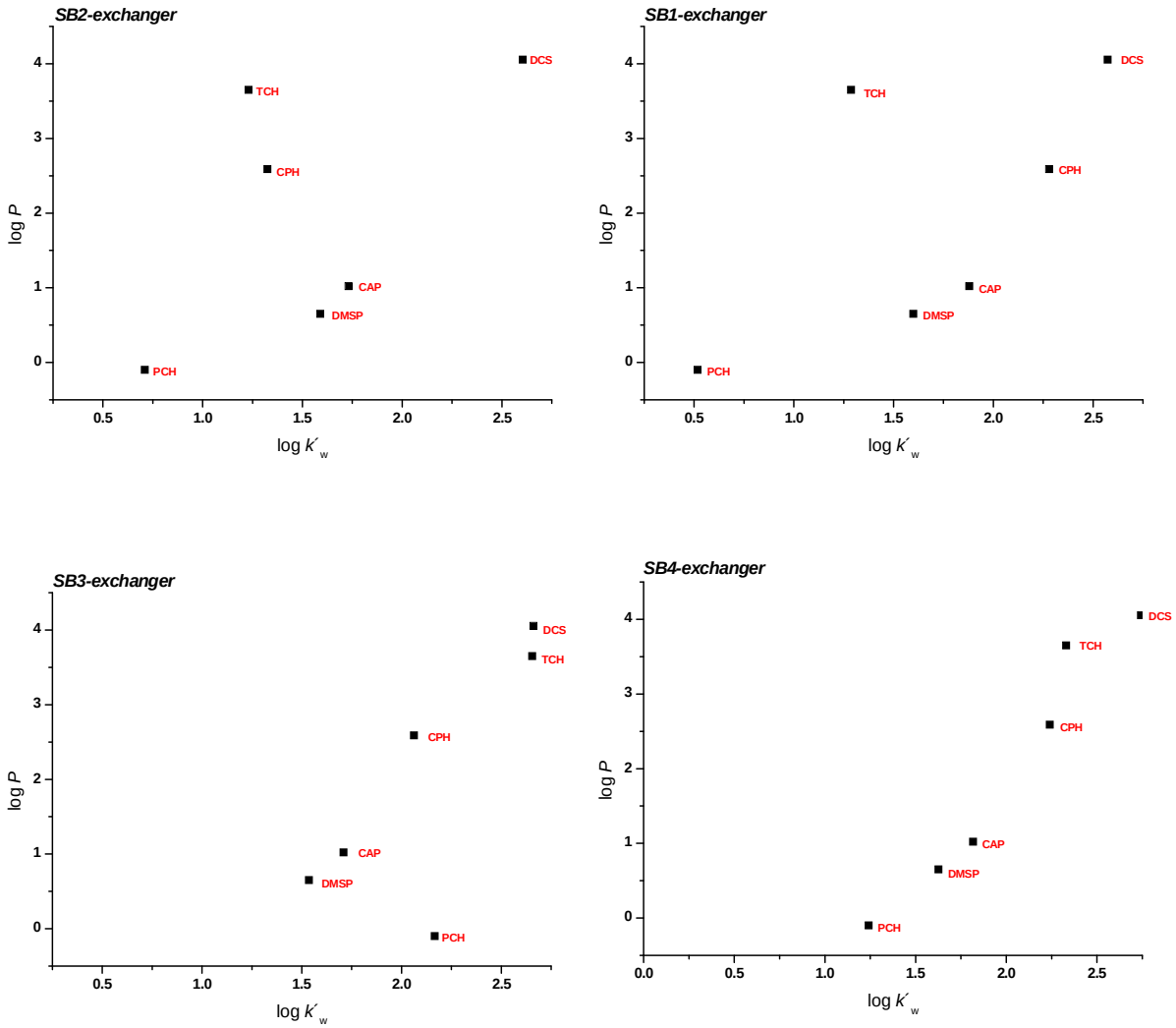


Figure 4.27: Relationship between *log k´w* and *log P* for six investigated compounds (CPH, DCS, DMSP, CAP, PCH and TCH) on SB_n-exchangers.

4.6.2.3 Variation of eluent concentration

ZIC-HILIC separations of test compounds with the prepared homologous row of exchangers exhibited mixed-mode separation mechanisms on most of the columns. The effect of salt concentration on the retention was also investigated by varying sodium acetate concentration from 20 to 80 mM in the mobile phase at constant acetonitrile (80%). The retention factor of DFOM, CPH, B1, PCH, TCH and CAP decreased with increasing buffer concentration.

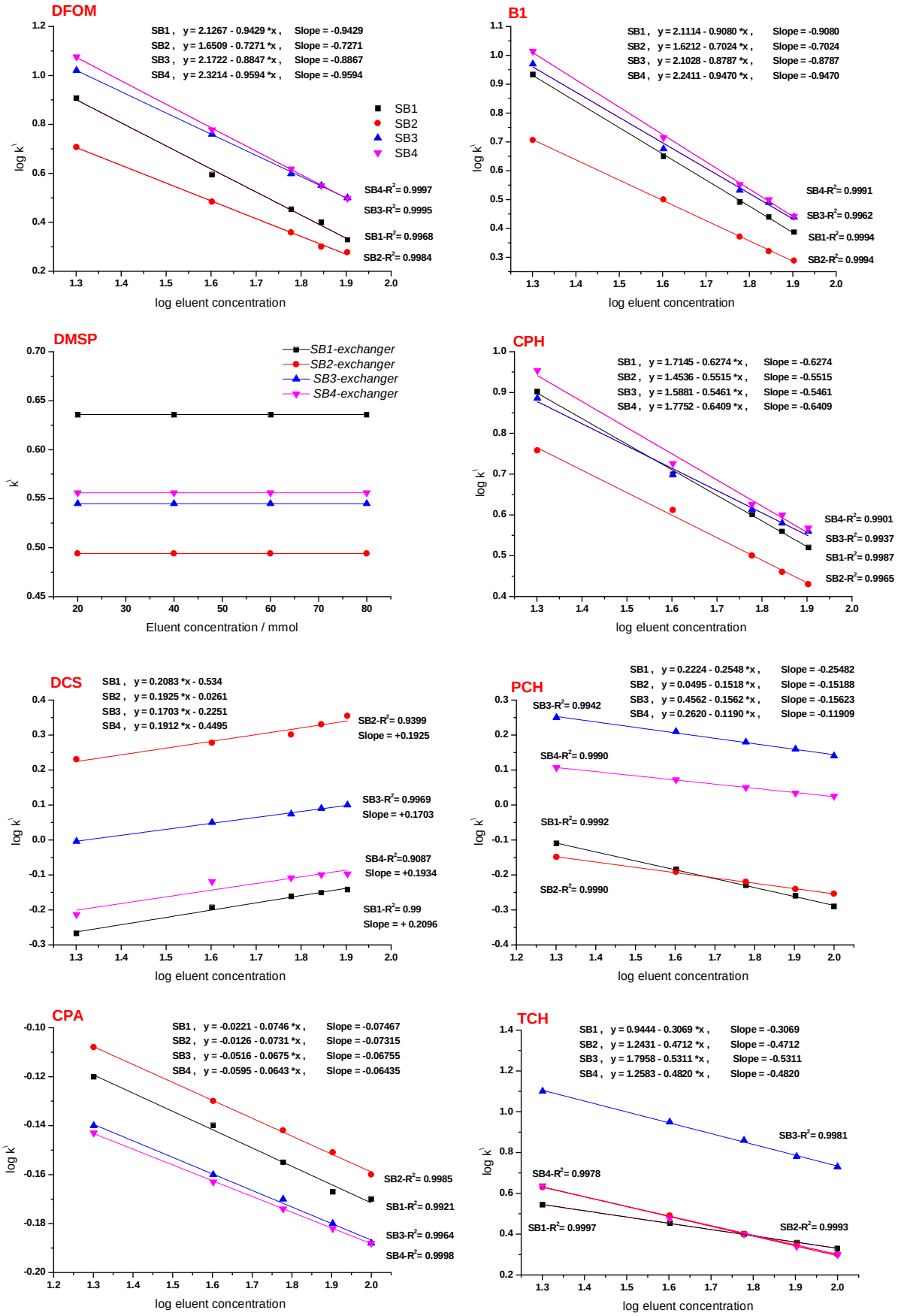


Figure 4.28: Variation of eluent ion concentration. Eluent: sodium acetate, pH 4.75, 80% acetonitrile; flow rate: 0.75 ml/min; temperature: 318 K; analyte concentration 50 mg/kg; using SB_n-columns.

The ion-exchange interactions on the ZIC-HILIC columns contributed significantly to the retention of the six model compounds. This can be seen from the decreasing retention with increasing buffer concentrations [135]. DCS exhibits an increase in the retention factor with an increasing buffer concentration in eluent. The reason for this could be an enhanced hydrophilicity of the enriched of the water layer leading to stronger retention of the analytes [135, 141, 142]. Interestingly, DMSP shows almost constant retention factors at quite high absolute values, so it may be attributed to the uncharged nature of DMSP. For a better understanding of the retention behavior of the compounds, one should have in mind the properties of the analytes. The pI's of the examined test compounds are between 0.96 and 11.30. The examined pH is 4.75, hence slightly below the pI of most of the pharmaceuticals investigated. Under the chosen conditions, all of the examined pharmaceuticals must be present in a more cationic state except the DCS and CAP, which should be in an anionic state (Figure 4.28). The slope of the plot of *log k´* versus log eluent concentration looks a like the plots found for standard ion exchange chromatography [43]. The upcoming question now is what the "true" separation mechanism is. Because of the simultaneous variation of anion and cation concentrations, it is impossible to judge if it is anionic, cationic or mixed mode ion exchange retention. For a deeper investigation of the HILIC retention mechanism, a set of anion and cation exchangers based on the same core material and using the same grafting reaction has been prepared. They use sulfonic acid or trimethylalkylammonium (TMA) as functional group [127]. Thus, the separation of the positively charged pharmaceuticals (DFOM, CPH, B1, PCH and TCH) and negatively charged pharmaceuticals (DCS and CAP) relied on the ion exchange with the stationary phase (Figure 4.29). This investigation provides additional evidence of an ion-exchange mechanism contributing to the separation of pharmaceutical compounds on zwitterionic stationary phases under HILIC mode conditions. Interestingly, no ion exchange effect has been observed for the DMSP on the anion and cation exchangers and four sulfobetaine exchangers due to that DMSP behaves like a neutral molecule. DMSP behavior cannot be explained by electrostatic interaction with functional groups on the zwitterionic stationary phases because of the similar behavior of DMSP on all exchangers and the retention perhaps only being related to hydrophobic interaction. In sum, the separation of the test compounds involved ionic interaction, hydrophilic and hydrophobic interactions.

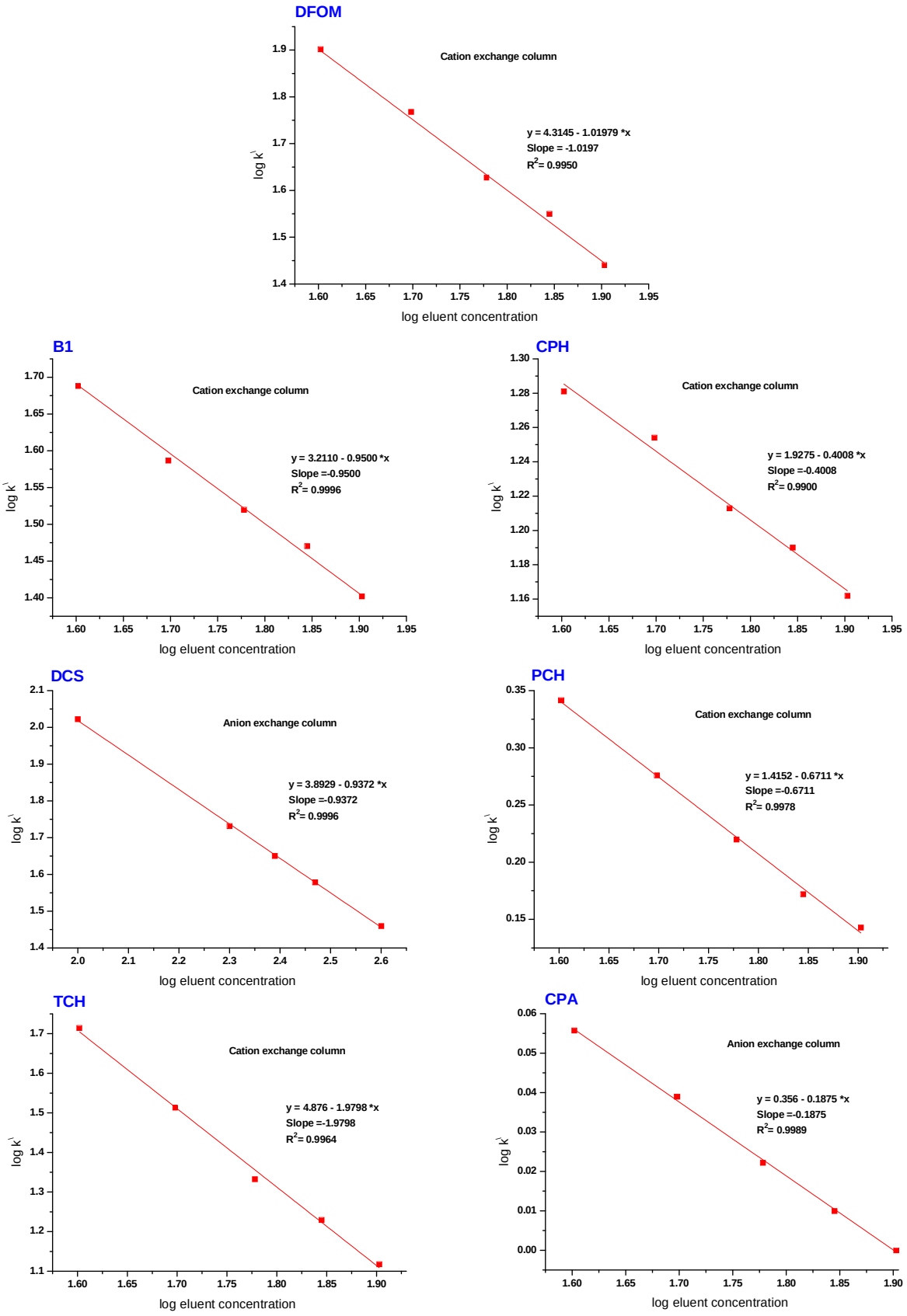


Figure 4.29: Variation of eluent ion concentration. Eluent: sodium acetate, pH 4.75, 80% acetonitrile; flow rate: 0.75 ml/min; temperature: 318 K; analyte concentration: 50 mg/kg; using ion exchange columns.

It turns out to us from Figure 4.30, the retention behavior of positive charge pharmaceuticals on anion exchanger and negative charge pharmaceuticals on cation exchanger are eluted all near the hold-up time. This behavior proves what has been pointed out previously, that the separation mechanism is a pure ion exchange mechanism.

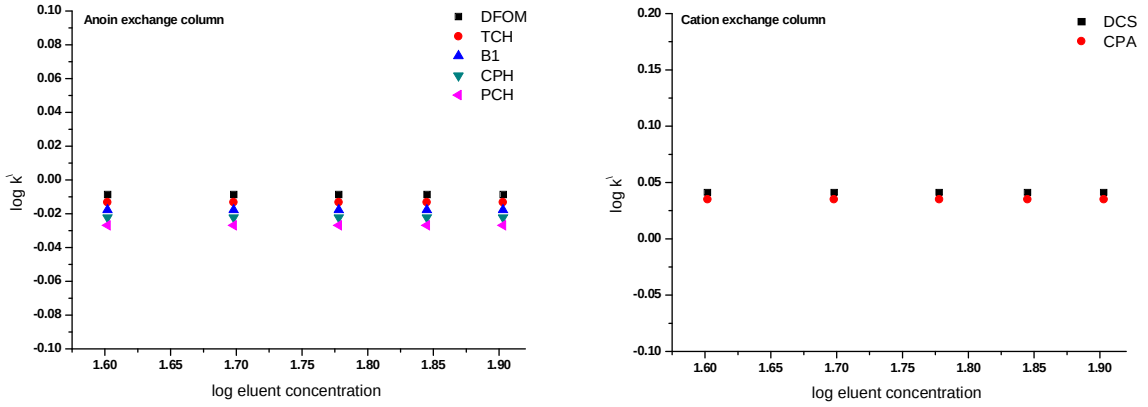


Figure 4.30: Variation of eluent ion concentration. Eluent: sodium acetate, pH 4.75, 80% acetonitrile; flow rate: 0.75 ml/min; temperature: 318 K; analyte concentration: 50 mg/kg; using ion exchange columns.

4.6.2.4 Variation of eluent pH

The pH of the mobile phase plays a significant role in controlling retention of the test compounds in HILIC by controlling the ionization state of the pharmaceuticals. The influence of pH was evaluated over the range from pH 4 to pH 5.5 (DFOM, B1, CPH, DCS and TCH) and from pH 3 to pH 5.5 (DMSP, PCH and CAP) at a constant buffer strength of 40 mM sodium acetate and constant acetonitrile fraction (80%) as shown in Figure 4.31. With regard to DFOM, CPH, B1 and TCH with a p*k*a ~7.9, 8.1, 3.8 and 8.2 respectively, the retention factors decreased on all the sulfobetaine columns due to the protonation of the amino group in DFOM, CPH, B1 and TCH in the pH range between 4 to 5.50. The pKa values were calculated with the program ACD/pKa (Table 4.7) [136]. For PCH with p*k*a ~ 7, the *k´* decreased in the investigated pH-range (pH 3-5.5).

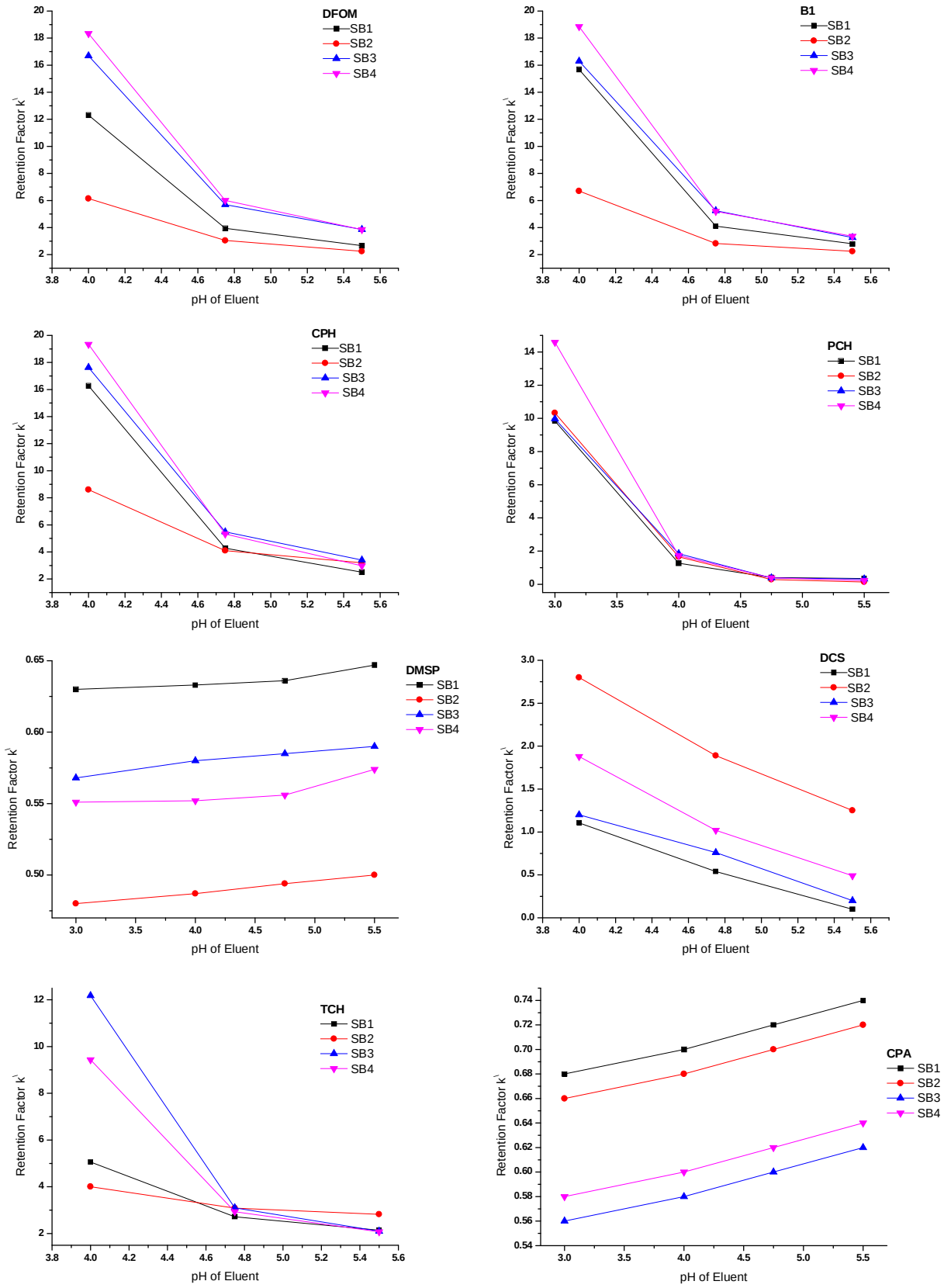


Figure 4.31: Variation of the pH of the eluent.Eluent:40 mM sodium acetate, 80% acetonitrile; flow rate: 0.75 ml/min; temperature: 318 K; analyte concentration: 50 mg/kg.

In sum, positively charged compounds (basic compounds) such as DFOM, B1, CPH, TCH and PCH show a decreasing retention with increasing eluent pH on sulfobetaine exchangers. DCS has a p*k*a ~4.4 and the deprotonation of the molecule increased with increasing pH from 4 to 5.5 leading to decreasing retention factors.

DMSP exhibits very slightly increase of the retention factors in the pH range (3-5.5) due to the unchanged charge (neutral state) of DMSP. At pH values between 3 to 5.5, chloramphenicol shows an anionic charge with pI~2.38 leading to increased k' values. Finally, we observed that the behavior of sulfobetaine exchangers is very similar while varying the acetonitrile content, the eluent concentration and the eluent pH.

4.6.3 Separation of metal–Deferoxamine complexes using zwitterionic ion chromatography (ZIC) coupled on-line with ICP-AES

Deferoxamine mesylate (DFOM, a pharmaceutical compound) is a strong chelator for multiple charged metal ions of biological importance (Fe^{3+}, Al^{3+}). The metal–DFOM complexes can be separated using the SB_n-columns in an IC-system and element specific detected using on-line coupling of the IC-system to ICP-AES. DFOM is a naturally-occurring trihydroxamic acid pharmaceutical with a terminal primary amine group [143] and is widely used for the removal of overload of iron from patients [144] and also used in the diagnosis and treatment of aluminium for haemodialysis patients [145].

DFOM is trihydroxamic ligand containing six oxygen-donors (three bidentate metals) and are capable of complete octahedral coordination to form a highly stable $M^{3+}L$ complex [143, 146]. DFOM has four protonation constants, the first three constants relate to the hydroxamate groups and the fourth to the terminal amino group. The four pK_a are associated to its structure as shown in Figure 4.32 [147, 148]. DFOM is in the physiological pH range which most of the time occurs in the fully protonated form of H_4DFOM.

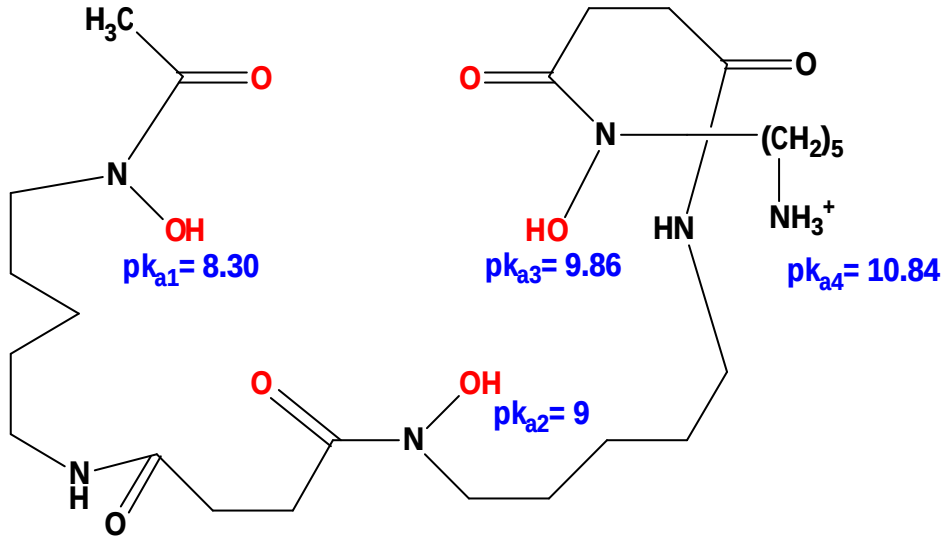


Figure 4.32: The structure of Deferoxamine (DFO).

DFOM has a high affinity towards multiple charged metal ions. Accordingly, it is used as a metal sequestering agent to treat certain metal overload diseases [143]. Due to its high affinity towards Fe(III) and Al(III), we investigated the separation the Fe(III)-DFOM and Al(III)-DFOM complexes using four zwitterionic stationary phases.

4.6.3.1 Separation Fe(III)-DFOM and Al(III)-DFOM complexes

Deferoxamine mesylate reacts with Fe(III) and Al(III) to form very stable complexes (Fe(III)-DFOM and Al(III)-DFOM) with stability constant of 10^{31} [149] and 10^{22} [150], respectively. The Fe(III)-DFOM and Al(III)-DFOM complexes separations are investigated, using sodium acetate eluents with 20% acetonitrile content. Retention of these complexes was observed for all of the sulfobetaine-exchangers.

In accordance with the a previous study [149], Deferoxamine has four protons, one from the protonated a terminal primary amine group and three from the hydroxamic acid group $(H_4\text{-DFOM})^+$. Therefore, the following metal-DFOM complex reactions are illustrated in Equations 4.3 [149] and 4.4 [151]:

$$H_4 - DFO^+ + Fe^{3+} \rightarrow 3H^+ + Fe\,(H - DFOM)^+ \quad (4.3)$$

$$H_4 - DFO^+ + Al^{3+} \rightarrow 3H^+ + Al\,(H - DFOM)^+ \quad (4.4)$$

According to many previous studies, the complex stoichiometry was 1:1 for both Fe(III)-DFOM [149] and Al(III)-DFOM [150], as shown in Figure 4.33. Suitable retention of the two metal-DFOM complexes was achieved with 20% acetonitrile and 80% and sodium acetate eluent at a concentration of 40 mM with a pH of 4.75. The chromatograms of the Fe(III)-DFOM and Al(III)-DFOM complexes are shown in Figure 4.34 and Figure 4.35, respectively. The SB4-exchanger showed the highest retention times ($t_{R,\,Fe}$ = 9.1, $t_{R,\,Al}$ = 8), and the SB2-exchanger showed the lowest retention times ($t_{R,\,Fe}$ = 2.8, $t_{R,\,Al}$ = 3.2) of Fe(III)-DFOM and Al(III)-DFOM complexes, respectively.

The dependence of metal-DFOM complex retention on the charge distance for the four-sulfobetaine exchangers is shown in Figure 4.36. The results obtained on the sulfobetaine exchangers are inconsistent with the correlations found in previous work for the separation of anions and amino acids [10, 125].

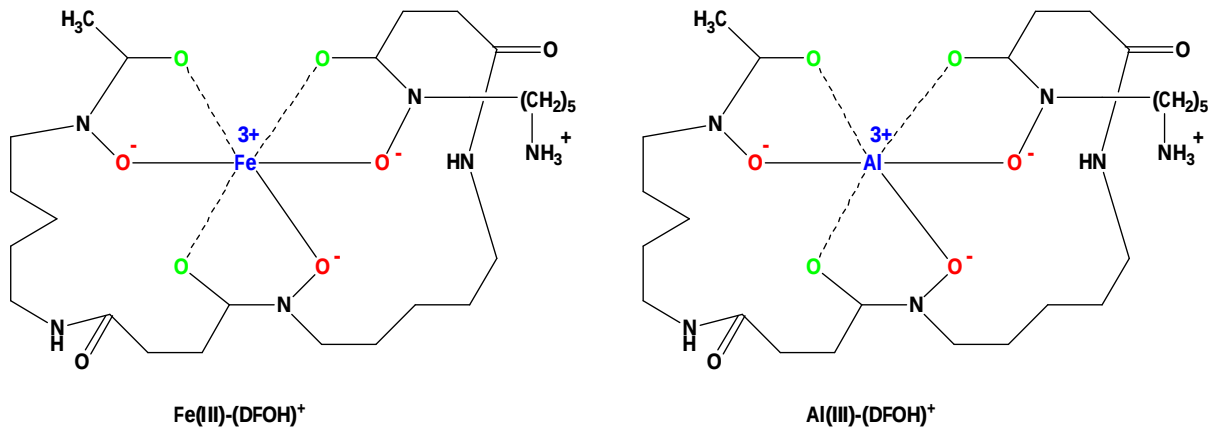


Figure 4.33: Probable chemical structures of the Fe(III)-DFO and Al(III)-DFO complexes.

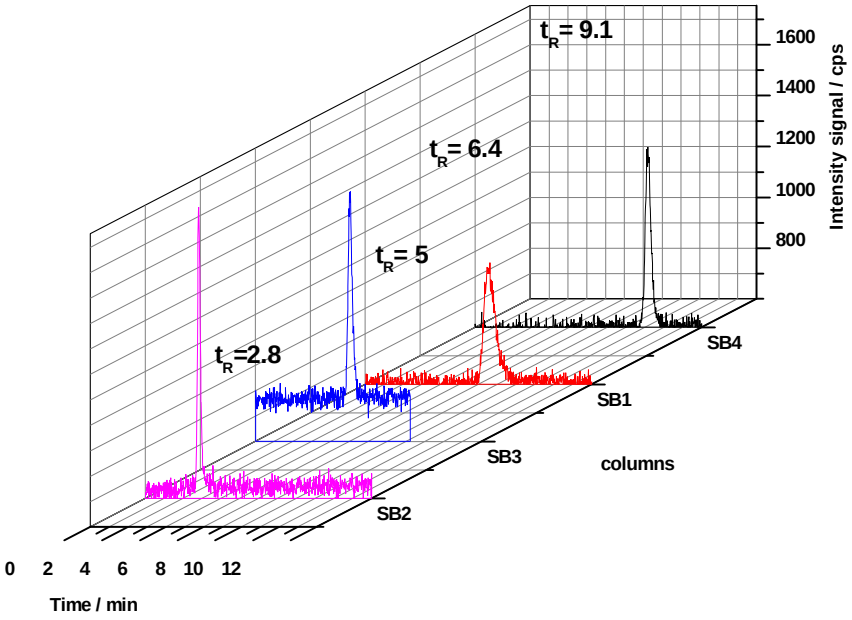


Figure 4.34: Chromatograms for the separations of Fe(III)-DFOM complex on SB_n-columns. Eluent: acetonitrile/sodium acetate buffer (pH4.75, 40 mM) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 259.940 nm, 318 k.

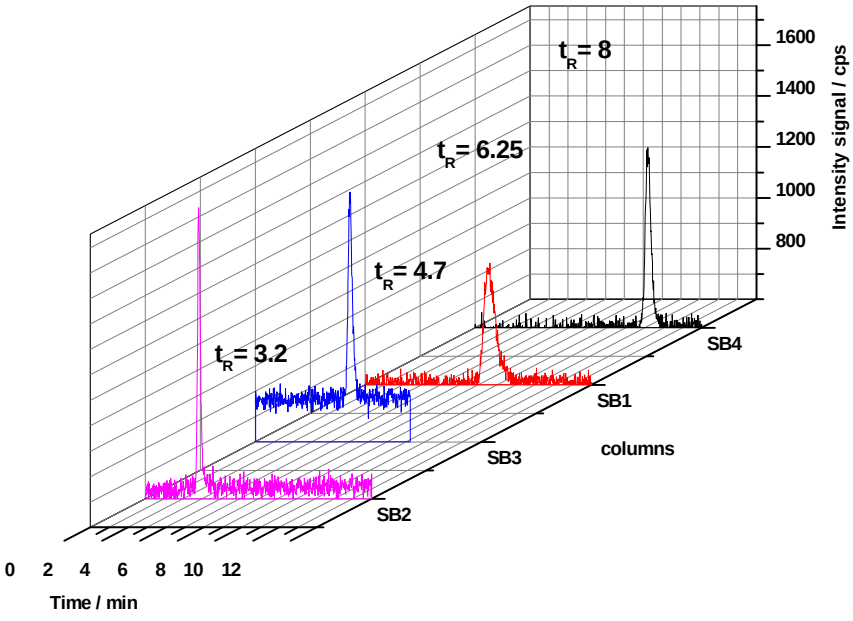


Figure 4.35: Chromatograms for the separations of Al(III)-DFOM complex on SB_n-columns. Eluent: acetonitrile/sodium acetate buffer (pH 4.75, 40 mM) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 396.152 nm, 318 k.

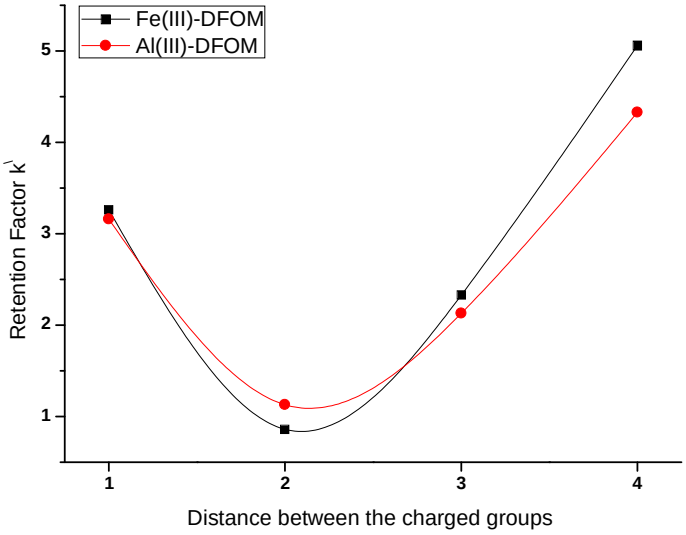


Figure 4.36: Dependency of metal-DFOM complex retention factors on the chain length between the charged groups. Eluent: acetonitrile/sodium acetate buffer (pH 4.75, 40 mM) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 259.940 nm (Fe) and 396.152 nm (Al).

The study of the influence of pH and eluent concentration of Fe(III)-DFOM and Al(III)-DFOM complexes selectivity should give a clue about the properties of the individual zwitterionic stationary phases and thus about the separation mechanism. It is worth mentioning that 20% acetonitrile is the upper limit before the plasma will be extinguished.

4.6.3.2 Influence of the eluent ionic strength on the separation of metal-DFOM complexes

The first step of eluent composition variation was the concentration of the sodium acetate buffer while holding the eluent pH and acetonitrile fraction constant. The influence of acetate concentration on the retention was investigated by varying sodium acetate concentration from 20 to 60 mM. The retention factors of Fe(III)-DFOM and Al(III)-DFOM complexes decreased with an increasing buffer concentration.

When the eluent concentration increased, the behavior of the metal-DFOM complexes was comparable to the behavior of the deferoxamine in Paragraph (4.6.2.1). Therefore, the ion-exchange interactions with the four-sulfobetaine exchangers contributed greatly to the retention of the two metal-DFOM complexes [135].

It should be noted all of the investigated metal-DFOM complexes must be present in a more cationic state [149, 150, 152]. The slope of the log-log-plots for changing eluent ionic strength of sodium acetate (Figure 4.37) looked like the plots observed for a pure ion exchange mechanism [43].

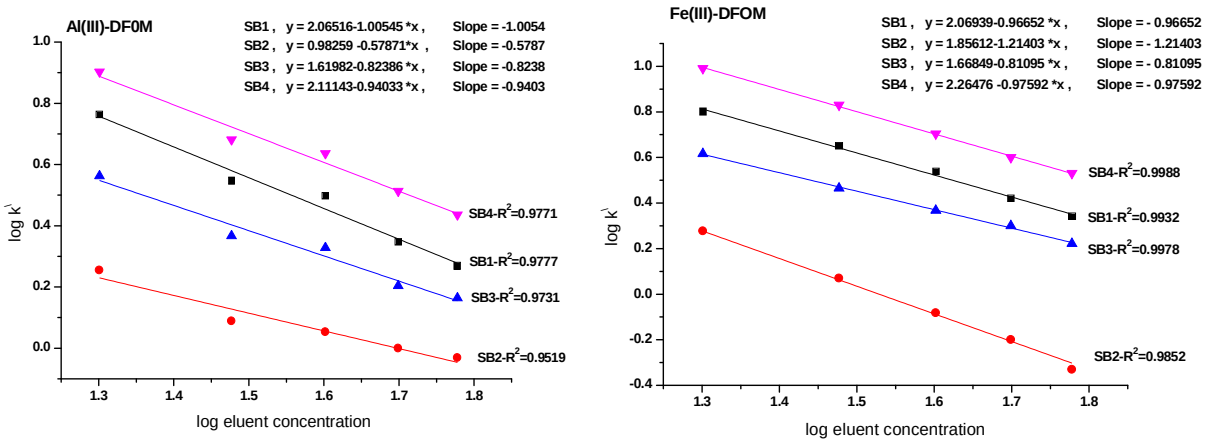


Figure 4.37: Influence of eluent concentration on the metal-DFOM complexes retentions for all examined exchangers. Eluent: acetonitrile/sodium acetate buffer (pH 4.75) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 259.940 nm (Fe) and 396.152 nm (Al), using SB_n-columns.

The question arising is what the true separation mechanism is. For this purpose a cation exchanger (Figure 3.38) based on the same core material and using the same grafting reaction [127] has been used. Accordingly, the separation of the positively charged of metal-DFOM complexes (Fe(III)-DFOM and Al(III)-DFOM) relied on the ion exchange with the four sulfobetaine stationary phases.

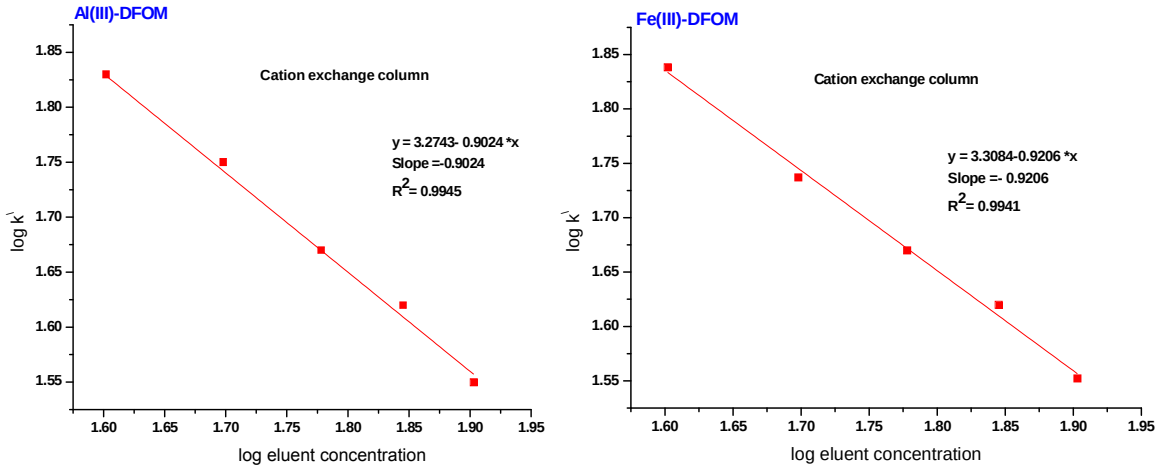


Figure 4.38: Influence of eluent concentration on the metal-DFOM complexes retentions for all examined exchangers. Eluent: acetonitrile/sodium acetate buffer (pH 4.75) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 259.940 nm (Fe) and 396.152 nm (Al), using cation exchange column.

4.6.3.3 Separation of metal-DFOM complexes with varying eluent pH

After having exhibited the influence of the eluent concentration for the metal-DFOM complex separations, the next variation in eluent composition is a variation of eluent pH. The pH of the eluent plays an important role for the retention of the metal-DFOM complexes by affecting metal-DFOM complexes ionization. The influence of pH was investigated over the range from pH 4 to pH 5.5 with 40 mM sodium acetate and constant acetonitrile fraction of 20% (Figure 4.39 and Figure 4.40). The retention of the Fe(III)-DFOM and Al(III)-DFOM complexes with their positive charge decreased on all the sulfobetaine exchangers when the pH increased in the range between 4 to 5.5. This can be attributed to a stronger protonation of the metal-DFOM complexes. In other words, a decreased pH of the eluent leads to a more positive charged metal-DFOM complex. Subsequently, more cation exchange interaction with zwitterionic surface takes place. In sum, it could be proved that cation exchange is the predominant separation mechanism for the metal-DFOM complex separations.

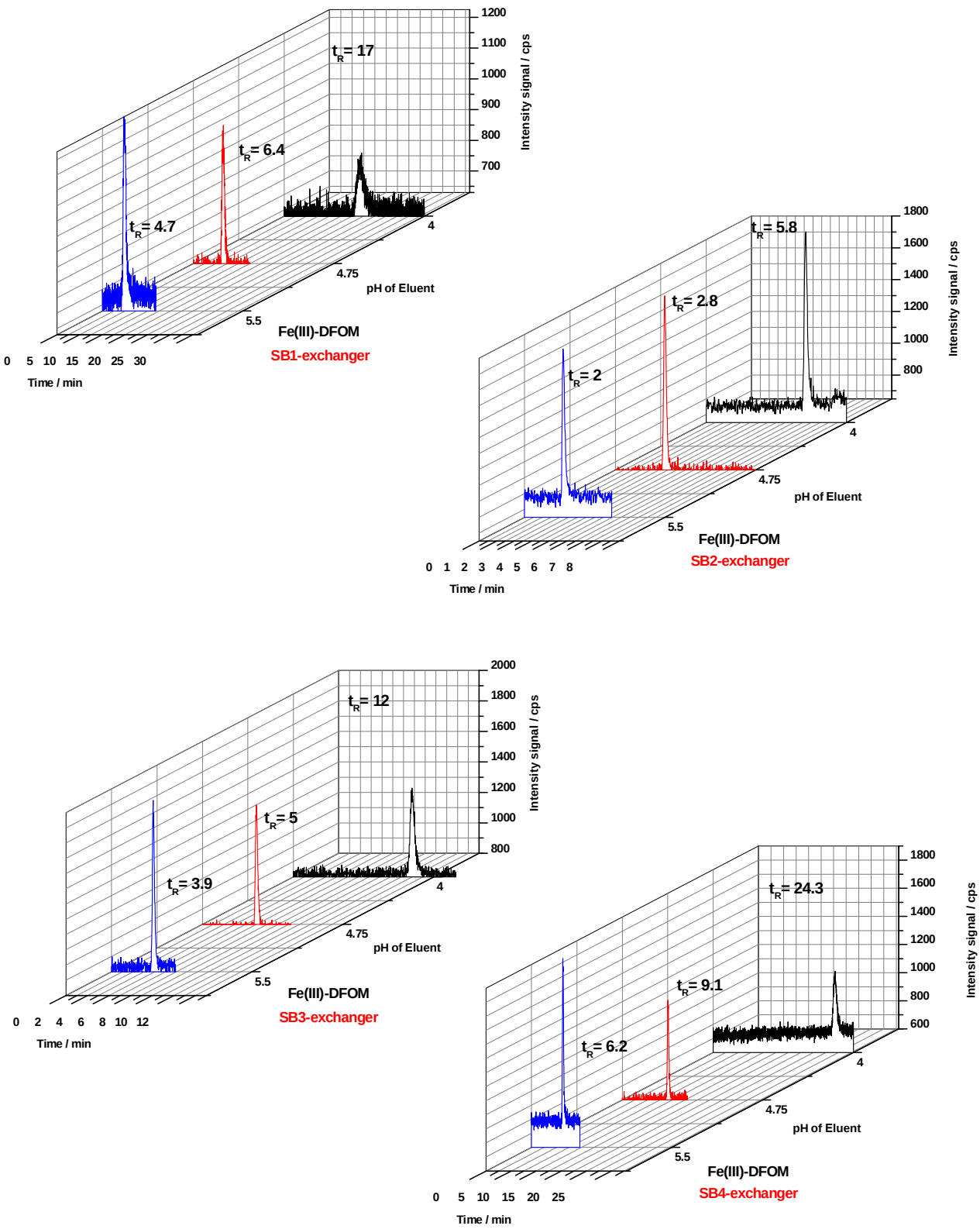


Figure 4.39: Influence of eluent pH on Fe(III)-DFOM retention. Eluent: acetonitrile/ sodium acetate buffer (40 mM) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 259.940 nm, 318 k.

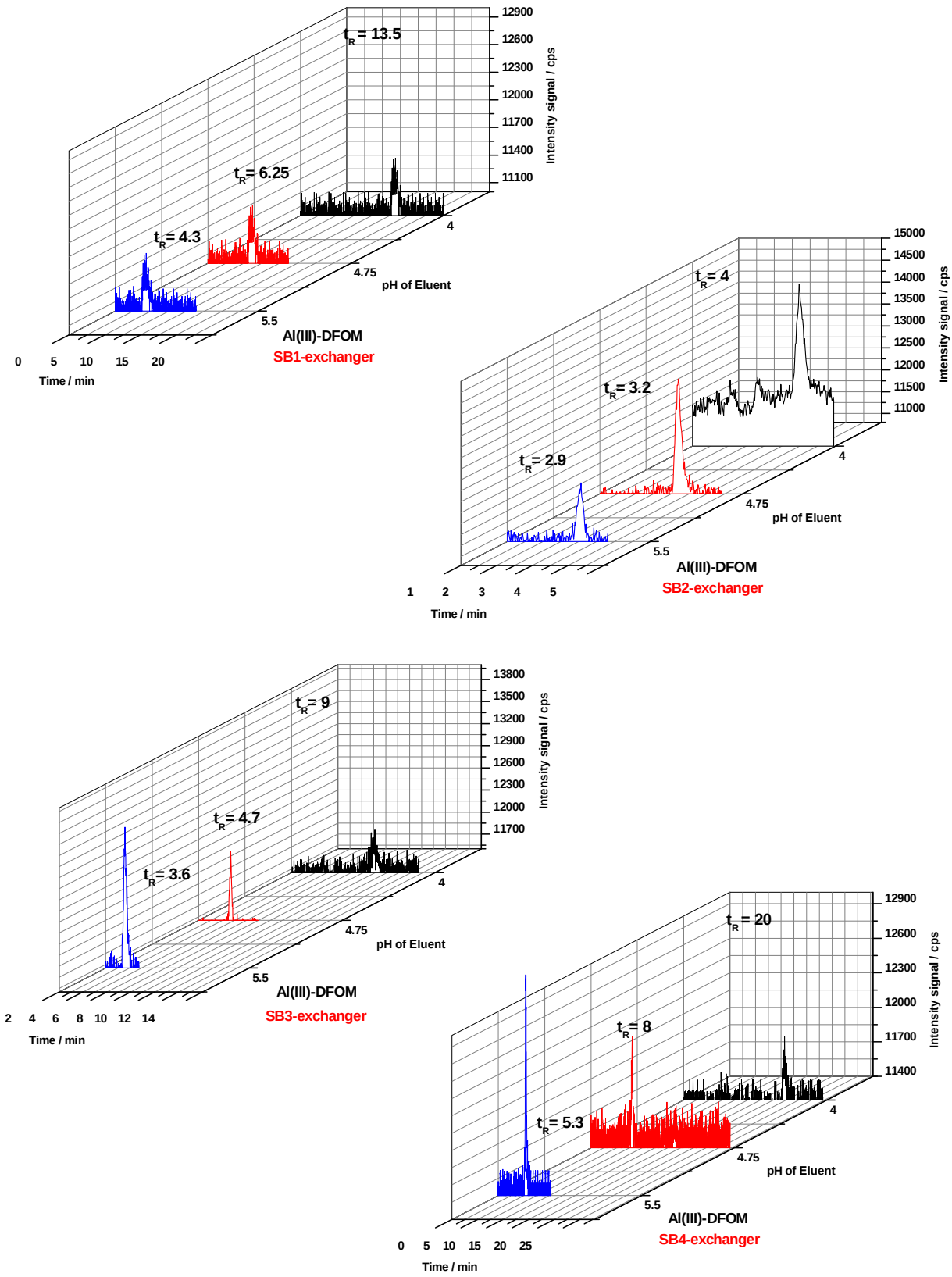


Figure 4.40: Influence of eluent pH on Al(III)-DFOM retention. Eluent: acetonitrile/sodium acetate (40 mM) 20:80; flow rate: 0.5 ml/min; detection: ICP-AES at 396.152 nm, 318 k.

4.7 Evaluation of excess adsorption isotherm of binary aqueous organic mixtures on various zwitterionic packing materials

In chromatography systems, adsorption is the process of the individual component accumulation on the adsorbent surface under the effect of the surface forces. In binary aqueous-organic mobile phases, component (A) has accumulated on the adsorbent surface and this is accompanied by displacement of another component (B) from the surface area into the bulk solution. At equilibrium, a certain quantity of the component has accumulated on the adsorbent surface in excess of its equilibrium concentration in the bulk solution [153]. In binary aqueous-organic eluent, *excess adsorption* Γ is defined from a comparison of two static systems: *firstly*, the adsorbent surface will be inactive and the total quantity of component A will be equal [154]:

$$n_0 = V_0 C_0 \tag{4.5}$$

Secondly, the adsorbent surface is active and the component A is adsorbed the total quantity of component A will be equal [154]:

$$n_e = V_0 C_e \tag{4.6}$$

Thereby, the amount of the component A in the bulk solution decreased. It is worth mentioning that the two systems have the same liquid volume V_0 and adsorbent surface area S. The original quantity n_0 of component A in Equation 4.5 is larger than the total quantity n_e in Equation 4.6 because of its accumulation on the adsorbent surface, so at equilibrium the component concentration C_e can only be measured in the bulk solution [153]. The excess adsorption Γ can be calculated from the difference between Equation 4.5 and Equation 4.6, and the excess quantity is related to the unit of the adsorbent surface and is given by:

$$\Gamma(C_e) = \frac{V_0}{S} (C_0 - C_e) \tag{4.7}$$

Figure 4.41 visualizes the excess adsorption system; the first system in Figure 4.41 (a) represents the inert adsorbent surface, C_0 is the original concentration of the component in the whole volume of the mobile phase before adsorption. The second system in Figure 4.41 (b) represents the active adsorbent surface; C_e is the equilibrium concentration of the component in the bulk solution (mobile phase) after adsorption [154].

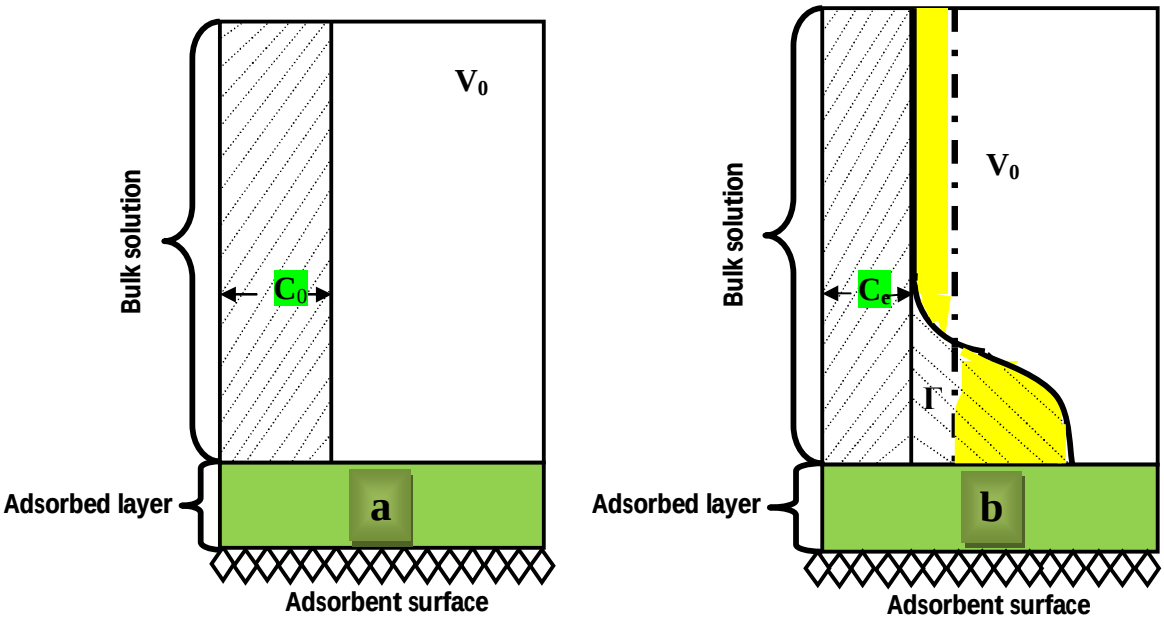


Figure 4.41: Conception of the excess adsorption system [154].

4.7.1 Excess isotherm calculation methodology

Nowadays, the minor disturbance method is the most commonly used method to measure the excess adsorption isotherms of the components of binary mixtures in liquid chromatography [21, 154-161]. The relationship of excess adsorption isotherm $\Gamma(c)$ and the component retention volume V_R in a binary dynamic adsorption system is expressed as:

$$V_R = V_0 + S.\frac{d\boldsymbol{\Gamma}(\boldsymbol{c})}{dc} \tag{4.8}$$

Here, V_0 is the thermodynamic void volume of the HPLC column, S is the adsorbent surface area. Detailed derivation of the essential equation for the determination of excess adsorption isotherms in LC (Equation 4.8) is given by *Kazakevich and McNair* [162]. The thermodynamic void volume V_0 (Equation 4.9) of the columns was calculated by integrating (from 0% to 100% of the solvent) the minor disturbance peak retention volumes [162]:

$$V_0 = \frac{1}{C_{ACN,H_2O}} \int_0^{C_{ACN,max}} V_R\left(c_{ACN,H_2O}\right) dc_{ACN,H_2O} \tag{4.9}$$

Excess adsorption isotherms were calculated by integration of the above Equation (4.8) and leads to Equation (4.10), which we used for the calculation of the excess adsorption isotherms from the minor disturbance peaks retention data [154]:

$$\Gamma_{ACN,H_2O}(C_{ACN,H_2O}) = \frac{1}{S} \int_0^{C_{ACN,H_2O}} \left(V_R\left(c_{ACN,H_2O}\right) - V_0\right) dc_{ACN,H_2O} \tag{4.10}$$

For more than 30 years, most of the previous studies have focused on the adsorption isotherm measurement on RP-stationary phases [19-22]. *Buntz et al.* [161] investigated excess adsorption isotherms of acetonitrile and methanol from water on eight reversed-phase commercial columns (Kinetex-C18, Acsentis-C18, Halo-C18, Gemini-C18, Xterra-C18, XBridge-C18, Allure-C18 and YMC-C18). They introduced the *standard adsorption isotherm* for RP-C18 columns by demonstrating significant similarity of the adsorption properties of all RP-C18 columns by comparing of the excess adsorption isotherms on all these columns (Figure 4.42).

The adsorbed layer of water is formed on the adsorbent surface of the polar stationary phase in HILIC. *Bocian et al.* [163] concluded that the presence of polar functional groups

causes the variation in the adsorption of acetonitrile and methanol on the adsorbent surface of the stationary phase.

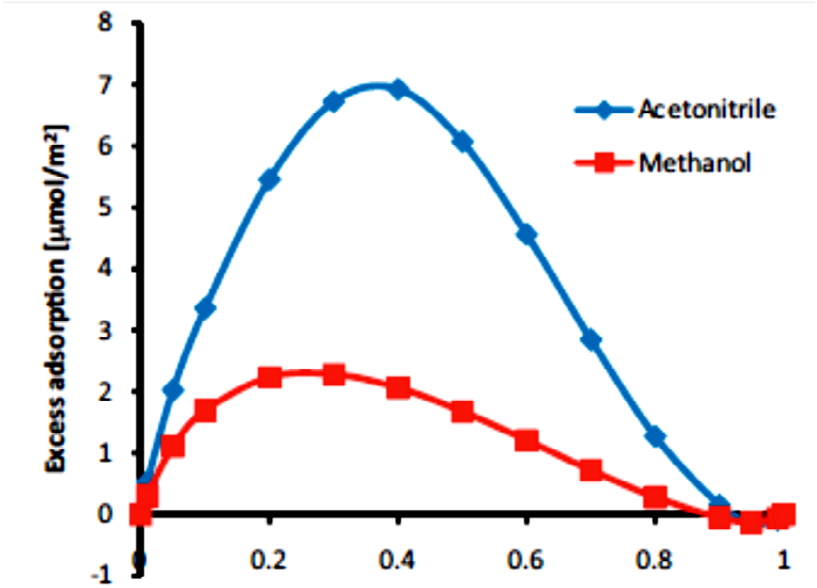


Figure 4.42: Standard excess adsorption isotherms of acetonitrile and methanol from water on RP- adsorbents shown by *Buntz et al.* [161].

It is important to investigate the excess adsorption isotherm of the eluent components on the surface of hydrophilic stationary phases. Recently, *Noga et al.* [164] had studied the excess adsorption isotherm of water from water–acetonitrile mixtures of surface of various types of HILIC-stationary phases with specific structural characteristics (Luna HILIC, TSK gel NH2-100, TSK gel Amide-80, Polyhydroxyethyl Aspartamide, Venusil Amide, Obelisc N, Obelisc R, Amino-C3, Diamino-C5, Amino-P-C10, AP-C2, AP-C12 and Amino-Chol). In their work, the excess adsorption of water is observed in acetonitrile-rich eluent and the excess adsorption of the acetonitrile is observed in the water-rich eluents.

In this work, ten zwitterionic stationary phases have been studied with the knowledge that all columns have inner quaternary amines and outer sulfonic acids. The zwitterionic stationary phases were divided into three groups: *The first group* includes eight columns (SB1-A, SB1-D and SB2-A, SB2-D and SB3-A, SB3-D and SB4-A, SB4-D).

Table 4.9: Physical-chemical characteristics of columns used in the investigations.

No.	Column	Dimension / mm	Particle size / µm	Pore diameter / Å	Surface area / $m^2 g^{-1}$	Capacity / $\mu eq\ g^{-1}$
1	SB1-A-EVO SB1-D-EVO	4 × 100	4.6	47	1073	296 669
2	SB2-A-EVO SB2-D-EVO	4 × 100	4.6	47	1073	300 790
3	SB3-A-EVO SB3-D-EVO	4 × 100	4.6	47	1073	212 281
4	SB4-A-EVO SB4-D-EVO	4 × 100	4.6	47	1073	250 288
5	SB1-ATRP	4 × 100	4.6	47	1140	1007 [165]
6	SB2-ATRP	4 × 100	4.6	47	1140	738 [165]
7	SB3-ATRP	4 × 100	4.6	47	1140	938 [165]
8	SB4-ATRP	4 × 100	4.6	47	1140	944 [165]
9	ZIC-HILIC	4.6 × 100	5	200	130	186 [10]
10	ZIC-pHILIC	4.6 × 100	5	100	-	201 [10]

It is noteworthy that the influence of exchangers' capacity is of special interest, so we chose the lowest and highest capacity for each sulfobetaine type.

The second group includes four columns (SB1-ATRP, SB2-ATRP, SB3-ATRP and SB4-ATRP) (*Note: Columns were prepared by my colleague Anna Teiz from the Seubert group*) using the same sulfobetaine molecules in the first step but a different functionalization procedure for the polymer particles. Anna Teiz used the functionalization of the PS/DVB-VBC via atom transfer radical polymerization-ATRP (second group) [165].

The third group includes two commercially available stationary phases (ZIC-HILIC and ZIC-pHILIC) from Merck SeQuant. It is worth mentioning that the core material in the first group is PS/DVB whereas it is PS/DVB-VBC for the second group. The ZIC-HILIC and ZIC-pHILIC columns have either silica or methacrylate cores and the commercially exchangers have three methylene groups between the charged functional groups (SB3). The characteristics of all the columns investigated in this study are summarized in Table 4.9.

In a chromatography system, water and acetonitrile are competing for the adsorption places surface. Consequently, adsorption of binary aqueous organic mixtures mobile phase is a competitive phenomenon due to surface heterogeneity of RP and HILIC stationary phases [164, 166]. Excess adsorption occurs due to two reasons: First, HILIC materials have hydrophilic, hydrophobic and ionic groups in the structures of the surface of the adsorbents. Second, the eluent composition varies over the whole range from pure water to pure organic modifier [164].

Figure 4.43 A-D shows the excess adsorption amounts (mmol/m^2) of water on the four stationary phases from the first group. It can be observed from Figure 4.43 that a large excess of water are adsorbed on SB4-exchangers.

SB1-EVO-exchanger exhibits the excess water adsorption on the adsorbent surface in a wide range of water concentration (0–30%). It is worth mentioning that we observed a huge negative part of excess isotherms due to the adsorption of acetonitrile.

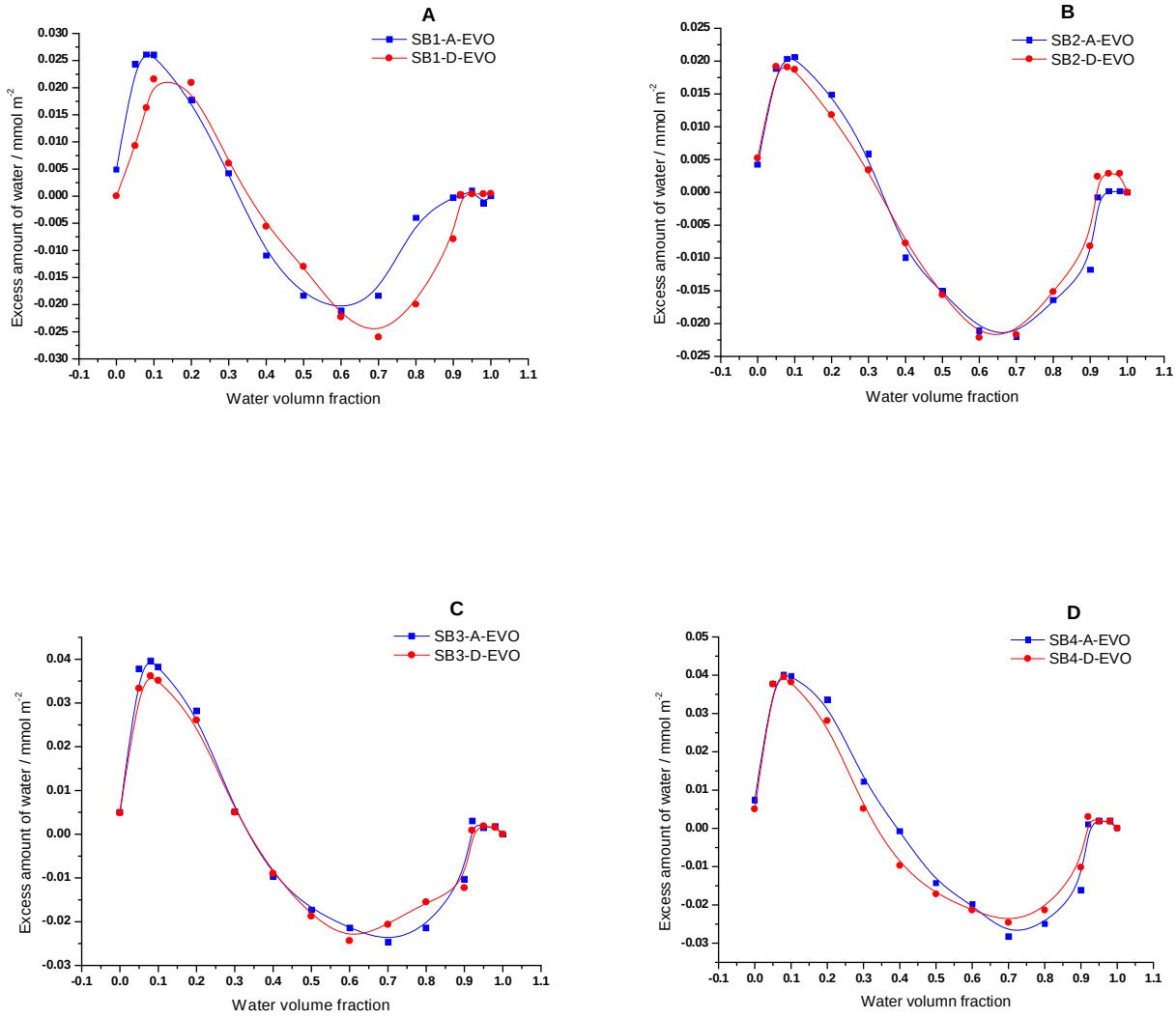


Figure 4.43: Excess adsorption isotherms of water from acetonitrile measured using the minor disturbance method (MDM) on a series of sulfobetaine stationary (functionalization of the resin was performed by a grafting reaction).

The results obtained on the other zwitterionic stationary phases (SB2-EVO, SB3-EVO and SB4-EVO) show a similar behavior than that observed for the SB1-exchanger (Figure 4.43 A-D). However, it should be noted that the values of the excess water adsorption on the adsorbent surface increased with increasing the chain length (methylene group) between the charged functional groups in the following order SB4-EVO > SB3-EVO > SB1-EVO > SB2-EVO.

Furthermore, when studying the excess adsorption isotherm of water at two capacities of each SB_{1-4}-column, it was observed, that the lower capacities of each type exhibit higher values for the excess adsorption of water than the higher capacity columns.

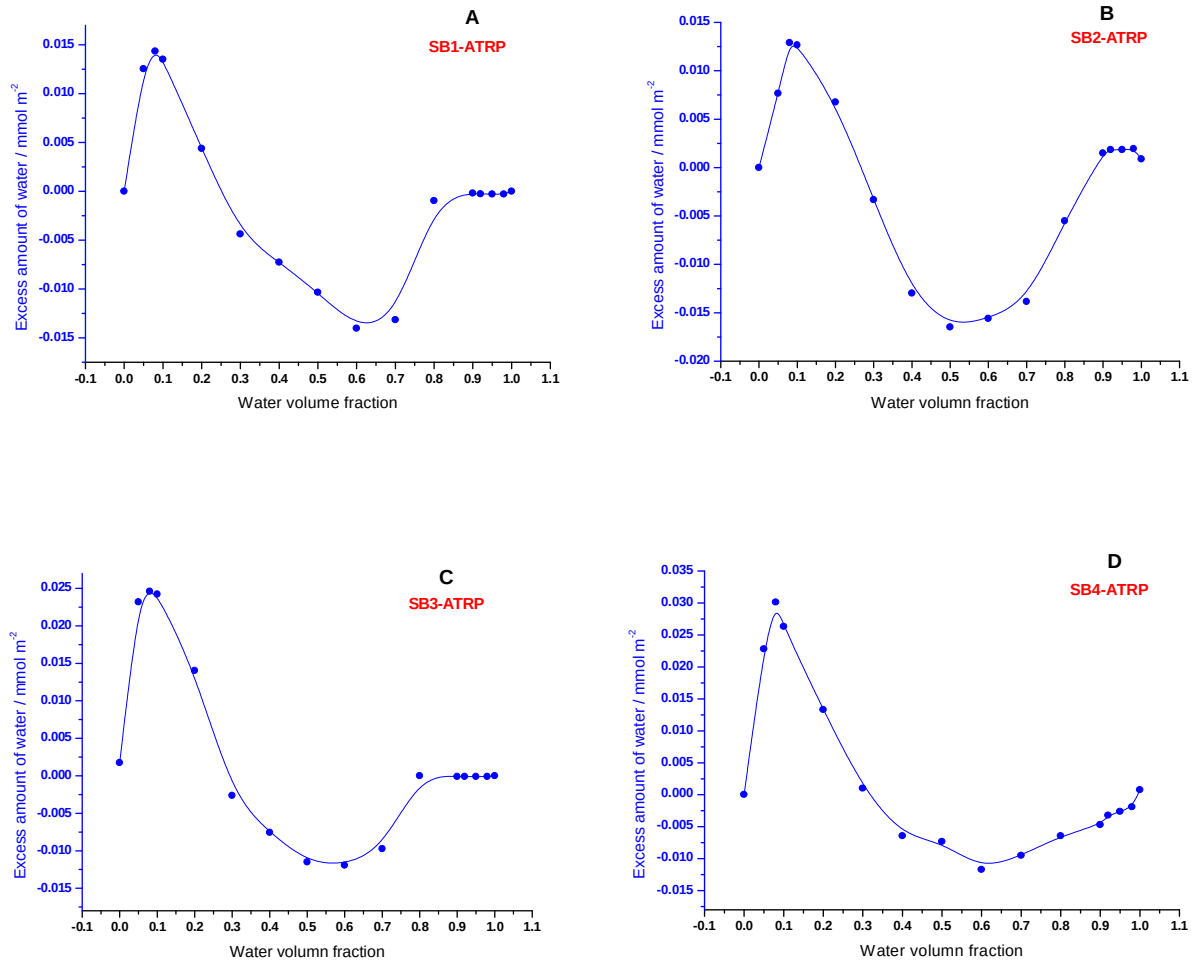


Figure 4.44: Excess adsorption isotherms of water from acetonitrile measured using the minor disturbance method (MDM) on a series of sulfobetaine stationary (functionalization of the resin was performed by an atom transfer radical polymerization).

Figure 4.44 A-D illustrates the water excess isotherms on a series of sulfobetaine stationary phases (SB_{1-4}-ATRP). Excess adsorption of water for all SB_{1-4}-ATRP exchangers is similar, but the SB2-ATRP exchanger exhibits the lowest value of excess adsorption of water.

It is worth mentioning that all SB_{1-4}-ATRP columns show the excess water adsorption on the adsorbent surface over a wider range of the water concentration (0–20%) in the eluent and that we observed a huger negative part of the excess which corresponds to adsorption of acetonitrile.

Moreover, it should be noted that the influence of the chain length between the quaternary amines and the sulfonic acids in the stationary phase is smaller to what has been observed in for the SB_{1-4}-EVO exchangers. As can be seen from Figure 4.45, the commercial ZIC-HILIC and ZIC-pHILIC columns show a high water adsorption (0–20%) and the large negative part of excess adsorption of acetonitrile is observed. Because of the unknown surface area of the ZIC-pHILIC column, the excess adsorbed amount is calculated per volume (mmol/ml $_{SP}$, V_{SP} is a volume of stationary phase in the column) of the stationary phase in the column.

For a comparison of all investigated columns, it was necessary to calculate the excess adsorption isotherm of water on the ZIC-HILIC column per volume and surface area. Finally, the results obtained for all stationary phases are compatible with previous findings [164].

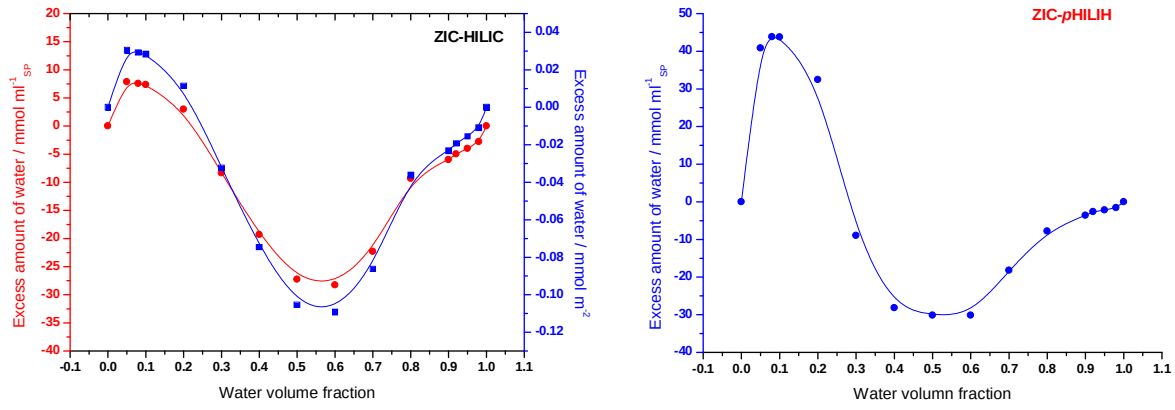


Figure 4.45: Excess adsorption isotherms of water from acetonitrile measured using the minor disturbance method (MDM) on the commercially ZIC-HILIC and ZIC-pHILIC exchangers.

It should be noted that a study measuring the excess adsorption isotherms of water on zwitterionic surface stationary phases from binary aqueous organic mixtures was according to three variables that had never been investigated previously: Firstly, the variable of chain

length between the charged functional groups, secondly, the variation of the column capacities and thirdly, the different functionalization methods of the polymer based resins. In conclusion, it could be shown and proven by increasing the concentration of the zwitterionic molecules on the PS/DVB surface that the result is a decrease of the water adsorption on the adsorbent surface. This can be attributed largely to increased inter- and intraolecular effects and, therefore, zwitterionic stationary phases exhibit a low tendency to adsorb water from binary aqueous organic mobile phase. Furthermore, we observed that increasing the distance (one to four methylene groups) between the amine and the sulfonic acid functionalities would lead to a higher tendency to extract water from the binary mobile phase.

The eight pharmaceutical compounds of chapter 4.6.3 (DFOM, B1, DCS, CPH, DMSP, TCH, PCH and CAP) were chosen as test analytes for a study of the correlation between the retention factors of analytes and the maximum excess of water adsorption (mmol). Initially, we have used binary water/acetonitrile (10:90) mixture mobile phase, but the analytes are eluted all near the hold-up time due to the presence of positive and negative charges in the zwitterionic molecules [164]. Therefore, we have used sodium acetate (40 mM-pH= 4.75) in the mobile phase at constant acetonitrile fraction (90%).

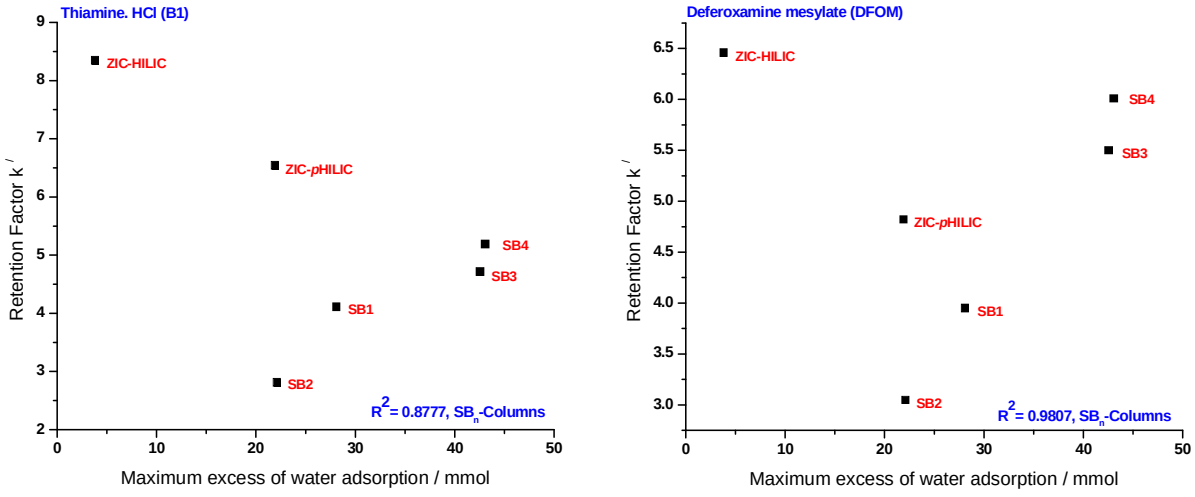


Figure 4.46: Relationship between the retention factor of the DFOM and B1 and maximum excess isotherm of sodium acetate in mobile phase: acetonitrile/ sodium acetate (90/10) containing 40mM sodium acetate (pH=4.75) on the series of SB_n-exchangers, ZIC-HILIC and ZIC-*p*HILIC columns.

Both, DFOM and B1 exhibit hydrophilic behavior (as discussed in paragraph 4.6.2). Figure 4.46 shows the maximum excess adsorption isotherms of water from acetonitrile on the sulfobetaine exchangers surface that are correlated with the retention factors of DFOM and B1. From Figure 4.46, we observed a good linear correlation for DFOM and B1 with R^2 values of 0.9807 and 0.8777, respectively.

It is worth mentioning that the R^2 values of DFOM and B1 for SB_n-columns decrease when adding the data from the commercial ZIC-HILIC and ZIC-*p*HILIC columns. In contrast, DCS, CPH, DMSP, TCH, PCH and CAP exhibit hydrophobic behaviors, the correlation is not good between the excess adsorption isotherms of water and the retention factors of analytes as shown in Figure 4.47 and that must be attributed to the RP behavior of those pharmaceuticals.

Noga et al. [164] have found a good linear correlation between the retention factors of the three analytes proline, tryptophan and glycine with the maximum excess of adsorbed water on 12 different types of HILIC stationary phases. The linear correlation depends significantly on the amount of water adsorbed on the HILIC surface. It should be noted that the presence of positive and negative charges in the zwitterionic structure (Obelisc R and Obelisc N) caused a stronger effect in the retention mechanism and, therefore, *Noga et al.* [164] have not used the zwitterionic stationary phases in their correlation comparison.

However, we have observed in our work that the correlation between the retention factors of eight pharmaceutical analytes (DFOM, B1, DCS, CPH, DMSP, TCH, PCH and CAP) and the maximum excess of adsorbed water on six zwitterionic stationary phases is not strong. The reason for this is due to the different behavior of the analytes, which means that interaction of pharmaceutical compounds with hydrophilic groups in the sulfobetaine surface play an important role in the retention mechanism in HILIC. In spite of the use of binary sodium acetate organic mixtures, SB1-ATRP, SB2-ATRP, SB3-ATRP and SB4-ATRP columns were not used in this comparison because the pharmaceutical compounds are all eluted near the hold-up time due to the high capacity of the columns caused by the presence of positive and negative charges in the zwitterionic molecules [164].

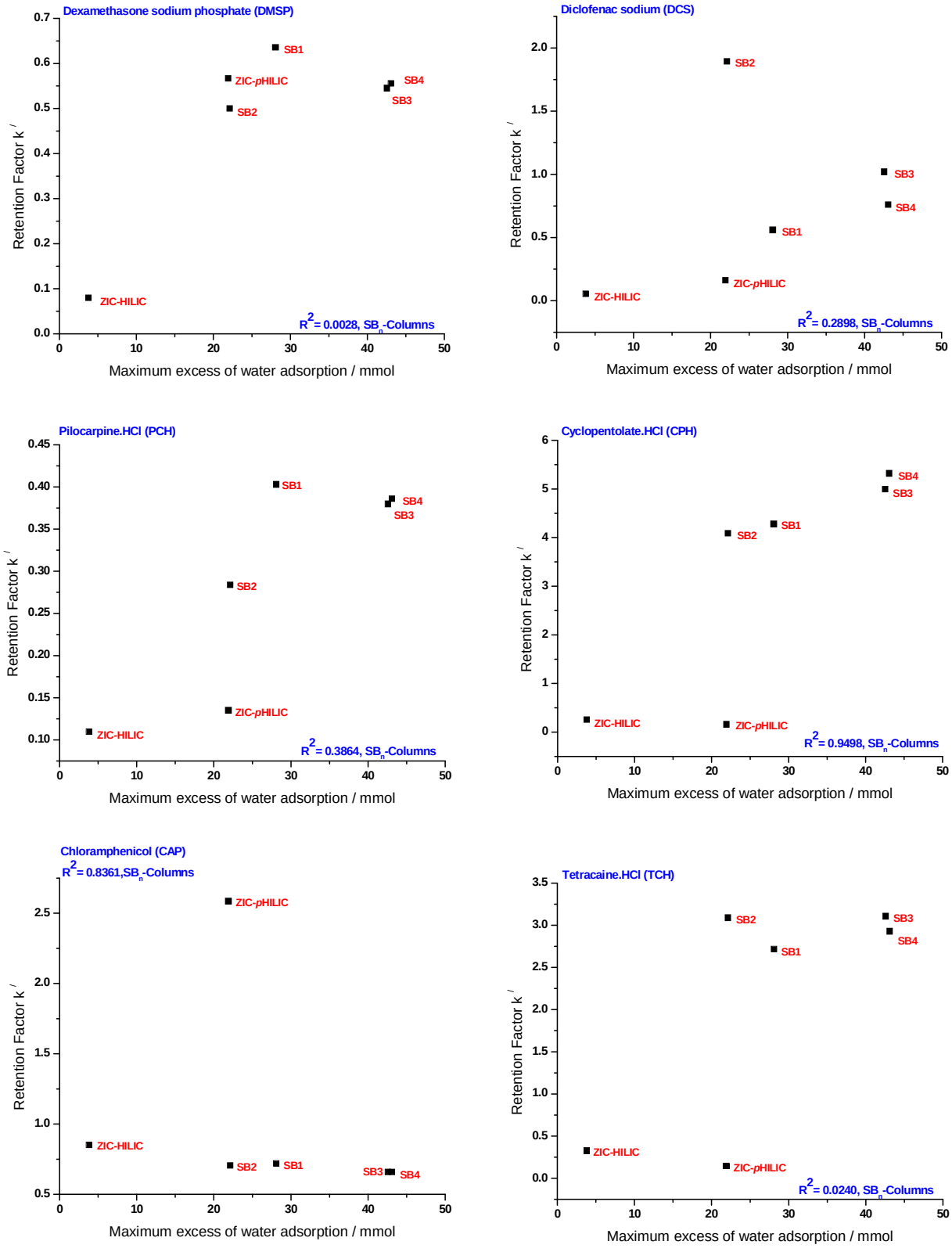


Figure 4.47: Relationship between the retention factor of the CPH, DCS, DMSP, CAP, PCH and TCH and maximum excess isotherm of sodium acetate in mobile phase: acetonitrile/ sodium acetate (90/10) containing 40mM sodium acetate (pH=4.75) on the series of SB_n-exchangers, ZIC-HILIC and ZIC-*p*HILIC columns.

5 Experimental

5.1 Chemicals

In the following tables, the chemicals and reagents used in this work were listed. Table 5.1 contains the all reagents for the synthesis of the zwitterionic precursors. The core material consisted of highly cross-linked macro porous polystyrene/divinylbenzene copolymer. The crosslinking degree was 55%, particle size was 4.6 µm (surface area (S) = 1073 m^2/g, mean pore diameter (φ_{50}) = 47 Å, specific pore volume (V_{Ps}) = 1.27 ml/g and total porosity (ε_T) = 70.5%). These data of the PS/DVB materials were measured by my colleague *Kristian Lungfiel* from the Seubert group.

Table 5.1: Chemicals and reagents used for the preparation of the stationary phases.

Term	Quality	Manufacturer	CAS-Nr.
Sodium hydroxymethanesulfonate	95%	Aldrich	870-72-4
N,N-dimethylamine	40% in water	Fluka	124-40-3
4-vinylbenzylchloride	≥ 90%	TCI Europe	1592-20-7
Ethanol	technical	-	64-17-5
Acetonitrile	gradient grade, ≥ 99.9%	Sigma-Aldrich	75-05-8
2-chloro-*N,N*-dimetylethylamine Hydrochloride	99%	Aldrich	4584-46-7
Sodium metabisulfite	p. a., ≥ 98%	Merck	7681-57-4
Lewatit S	highly acidic	Lanxess	-
4-vinylbenzyl-*N,N*-dimethyl amine	90%	Acros Organics	2245-52-5

Term	Quality	Manufacturer	CAS-Nr.
1,3-propanesultone	98%	Aldrich	1120-71-4
4-tert-butylcatechol	for synthesis,>98%	Merck	98-29-3
1,4-butanesultone	≥ 99%	Acros Organics	98-29-3
Potassium persulfate	p. a., ≥ 99,0%	Merck	7727-21-1
Acetone	technical	-	75-05-8
1,2–Dichloro propane	purum, ≥ 98,5%	Fluka	78-87-5
Toluene	technical	-	108-88-3
ammonia solution	p. a., 25%	Merck	1336-21-6
4-tert-butylcatechol	for synthesis,>98%	Merck	98-29-3
1,4-butanesultone	≥ 99%	Acros Organics	98-29-3

All reagent solutions were prepared using deionised Milli-Q water (Millipore, Bedford, MA, USA). The inorganic ions (used as the targeted ions in this study) were prepared by dissolving inorganic salts in pure water. The eluents were prepared by dissolving directly in ultrapure water for ZIC and ZIC-HILIC modes. Eluents and stock solutions of pharmaceutical compounds were prepared by dissolving directly in ultrapure water as shown in Tables (5-2) and (5-3).

Table 5.2: Chemicals and reagents used for the preparation of eluents and stock solutions of anions for ZIC and ZIC-HILIC separations.

Term	Quality	Manufacturer	CAS-Nr.
Acetic acid	p. a., 100%	Carl Roth	64-19-7
Sodium acetate	≥ 99%	J. T. Baker	127-09-3
Sodium nitrate	pure, 99%	Grüssing	7631-99-4
Sodium bromide	p. a., ≥ 99,0%	Fluka	7647-15-6
Sodium iodide	p. a., ≥ 99,5%	Merck	7681-82-5
Sodium thiocyanate	pure	Merck	540-72-7
Sodium nitrite	p. a., ≥ 99%	Merck	7632-00-0
Acetonitrile	gradient grade, ≥ 99.9%	VWR	75-05-8

Table 5.3: Chemicals and reagents used for the preparation of stock solutions of pharmaceutical compounds for ZIC-HILIC separations.

Pharmaceuticals	Quality	Manufacturer	CAS-Nr.
Deferoxamine Mesylate salt	≥ 92.5% (TLC)	Sigma	138-14-7
Cyclopentolate .HCl	analytical standard, for drug analysis	Fluka	5870-29-1
Diclofenac sodium salt	-	Sigma	15307-79-6
Thiamine .HCl	reagent grade, ≥99%	Sigma-Aldrich	67-03-8
Dexamethasone sodium phosphate	≥ 98%	Sigma	2392-39-4
Tetracaine .HCl	≥ 99%	Sigma	136-47-0
Pilocarpine.HCl	≥ 98%	Sigma	54-71-7
Chloramphenicol	≥ 98%	Sigma	56-75-7
Sodium metavanadate	≥ 98%	Sigma	13718-26-8
Iron nitrate nonahydrate	p. a., ≥ 97%	Riedel-de Haën	7782-61.8
Aluminum Nitrate nonahydrate	p. a., ≥ 98%	Merck	7784-27-2
Sodium hydroxide	p. a., 50%	Fluka	1310-73-2
Hydrochloric acid	p. a., 37%	KMF	7647-01-0

5.2 Synthesis of zwitterionic monomers

The procedures for the synthesis were adopted from previous work [126]. As the first step for my Ph.D. work, the desired zwitterionic monomers with the different distances between the charged groups were prepared and isolated as pure substances. The structures of the zwitterionic monomers SB1 to SB4 having sulfobetaine substructures were summarized in Figure 5.1. Nucleophilic substitution reactions between a monomeric spacer and tertiary amines were used for the synthesis of these molecules.

I reproduced the synthesis and generated enough substance for the sulfobetaine monomers SB1 to SB4. The detailed procedures as well as my quality control work related to the synthesis were as follows.

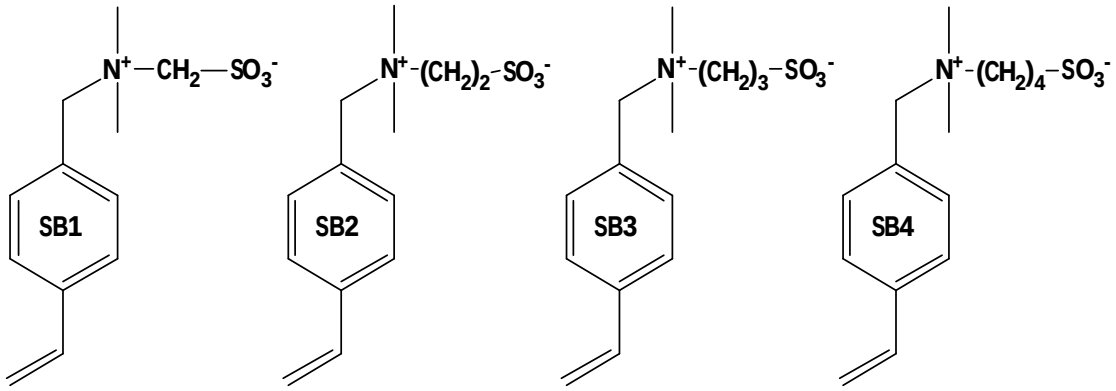


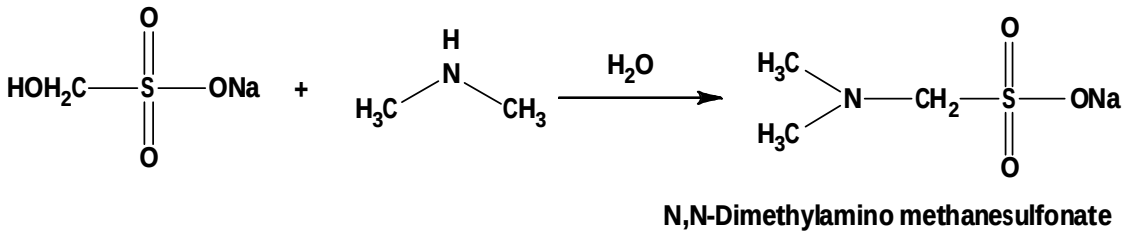
Figure 5.1: Structure of the synthesized sulfobetaine monomers SB1 to SB4.

5.2.1 Preparation of the sulfobetaine monomer SB1

5.2.1.1 Synthesis of N,N-dimethylamino methanesulfonate

N,N-dimethylamino methanesulfonate was prepared according to a procedure published by *King* and *Skonieczny* [128] starting from sodium hydroxymethanesulfonate and an aqueous solution of *N,N*-dimethylamine. 5.4 g of sodium hydroxymethanesulfonate (0.040 mol, 134.09 g/mol, 1.0 equiv) were dissolved in 20 ml of water and cooled in an ice bath. Over a period of 30 min, through a dropping funnel 5.88 ml of *N,N*-dimethylamine (0.046 mol, 45.08 g/mol, 40% in water, 5.18 g, 1.15 equiv) were added to the solution. The ice bath was removed and it is stirred for 1 h at room temperature.

Water and excess *N,N*-dimethylamine were removed under reduced pressure and the product was dried under high vacuum. This gives 6.11 g of pure product as a colorless powder, which corresponds to yield of 94%.



N,N-Dimethylamino methanesulfonate

Figure 5.2: Synthesis of *N,N*-dimethylamino methanesulfonate [126].

^{1}H-NMR (D_2O, 300 MHz): 3.74 (s, 2H, CH_2); 2.49 (s, 6H, 2 × CH_3).

5.2.1.2 Synthesis of 4-vinylbenzyl-dimethylammonio methanesulfonate SB1

The 4-vinylbenzyl-dimethylammoniomethansulfonat was prepared analogous to the procedure developed by *Raskop et al.* [130] by reaction of 4-vinyl benzyl chloride with a tertiary amines. In an inert gas filled two-neck flask, 2.82 ml of 4-vinylbenzyl chloride (0.020 mol, 152.62 g/mol, 3.05 g, 1.0 equiv) were dissolved in 20 ml of ethanol and cooled in an ice bath. A solution of 3.22 g *N,N*-dimethylamino methanesulfonate (0.020 mol, 161.16 g/mol, 1.0 equiv) in 50 ml ethanol/water (1:1) were added dropwise over a period of 15 minutes. The reaction mixture was heated for 24 h at 318 k.

Then the solvent was removed under reduced pressure and the product was washed with acetonitrile to give 6.12 g of SB1 as a colorless powder which corresponds to yield of 97%. The product was stored under nitrogen at 248 k.

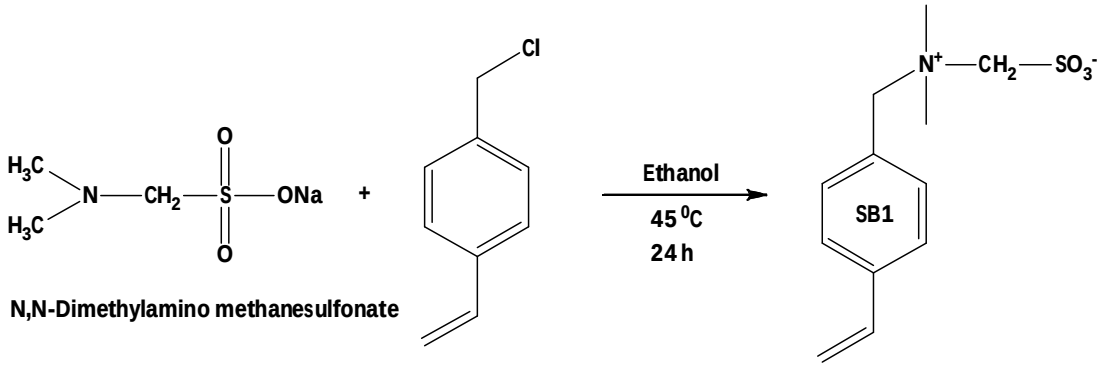


Figure 5.3: Synthesis of 4-vinylbenzyl-dimethylammonio methanesulfonate SB1 [126].

^{1}H NMR (300 MHz, D_2O): 7.57 (m, 4H, CH_{aromat}); 6.81 (dd, 1H, ^3J = 11.0 Hz and 17.7 Hz, CH_{olefin}); 5.93 (d, 1H, ^3J = 17.7 Hz, CH_{trans}); 5.40 (d, 1H, ^3J = 11.0 Hz, CH_{cis}); 4.73 (s, 2H, $CH_{2,\ benzyl}$); 4.41 (s, 2H, CH_2); 3.26 (s, 6H, 2 × CH_3).

Elemental analysis: *calculated*: $C_{12}H_{17}NO_3S$: C 45.9%; H 5.5%; N 4.5%. ***found***: C 44.8%; H 5.7%; N 4.0%.

5.2.2 Preparation of the sulfobetaine monomer SB2

5.2.2.1 Synthesis N,N-dimethyltaurine hydrochloride

The preparation of *N,N*-dimethyltaurine hydrochloride was carried out according to a procedure by *Palmi et al*. [129] by the reaction of 2-Chloro-*N,N*-dimethylamine hydrochloride with sodium metbisulfate. In a round bottom flask with intensive condenser, a solution of 13.31 g sodium metbisulfate (0.070 mol, 190.10 g/mol, 2.0 equiv) in 80 ml water and 10.08 g 2-chloro-*N,N*-dimethylamine hydrochloride (0.070 mole, 144.04 g/mol, 1.0 equiv) was poured.

The reaction mixture was heated to boiling for 4 h and then cooled to room temperature. The excess sodium metabisulfite was removed using a strongly acidic cation exchange resin in the H^+-form [126]. For this purpose, 60 g Lewatit S was added to the reaction mixture.

The solvent and the SO_2 formed were removed under reduced pressure. Thereafter, the resin filtered off on a glass frit and washed with 150 ml acetic acid (2 mol/l). The eluate was collected and the acetic acid was removed under reduced pressure. This gives 10.77 g of pure product as a colorless powder, which corresponds to yield of 72%.

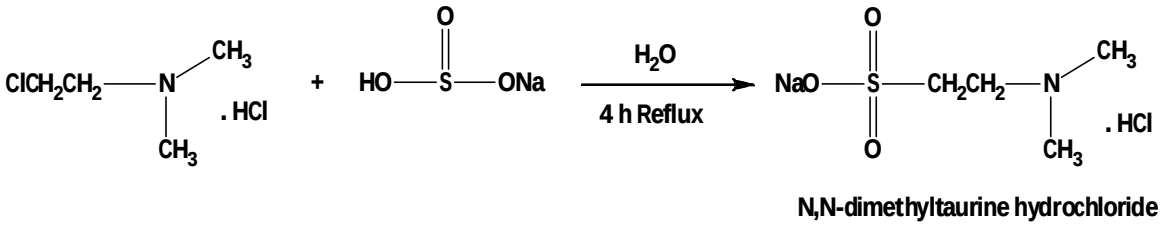


Figure 5.4: Synthesis of *N,N*-dimethyltaurine hydrochloride [126].

^{1}H-NMR (D_2O, 300 MHz): 3.63 (t, 2H, ^3J = 7.2 Hz, SCH_2); 3.44 (t, 2H, ^3J = 7.2 Hz, NCH_2) ; 3.03 (s, 6H, 2 × CH_3).

5.2.2.2 Synthesis of 4-vinylbenzyl-dimethylammonio ethanesulfonate SB2

The preparation of 4-vinylbenzyl-dimethylammonio ethanesulfonate (SB2) was also possible analogous to that route shown by *Raskop et al.* [130] by reaction of *N,N*-dimethyltaurine hydrochloride with 4-vinylbenzyl chloride. Under nitrogen atmosphere 10.69 ml of 4-vinylbenzyl chloride (0.076 mol, 152.62 g/mol, 11.57g 1.1 equiv) were dissolved in 30 ml of ethanol and cooled in an ice bath.

To this solution, a solution of 12.0 g *N,N*- dimethyltaurine hydrochloride (0.056 mol, 211.64 g/mol, 1.0 equiv) in 180 ml of ethanol/water (2:1) was added dropwise over a period of 15 minutes. Then 9 ml ammonia solution (25%) was added by pipette.

The reaction mixture was heated for 18 h at 323 k. The solvent was removed under reduced pressure. The residue was washed several times with acetonitrile to remove the excess of 4-vinylbenzyl chloride. There are obtained 16.5 g of colorless powder, which corresponds to a yield of 88%. The product was stored under inert gas atmosphere at 248 k.

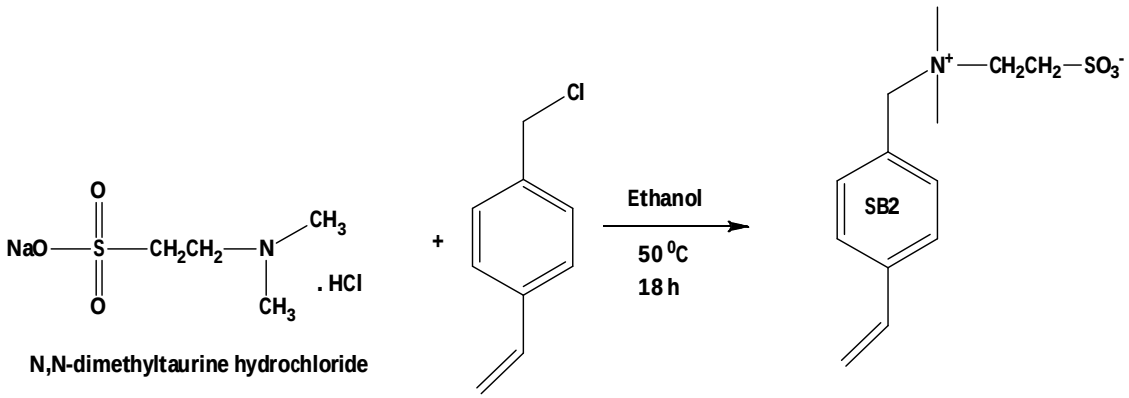


Figure 5.5: Synthesis of 4-vinylbenzyl-dimethylammonio ethanesulfonate SB2 [126].

^{1}H NMR (300 MHz, D_2O): 7.47 (m, 4H, CH_{aromat}); 6.68 (dd, 1H, ^3J = 11.0 Hz and 17.7 Hz, CH_{olefin});5.81 (d, 1H, ^3J = 17.7 Hz, CH_{trans}); 5.28 (d, 1H, ^3J = 11.0 Hz, CH_{cis}); 4.48 (s, 2H, $CH_{2,benzyl}$); 3.60 (m,2H, SCH_2); 3.38 (m, 2H, NCH_2); 3,01 (s, 6H, 2 × CH_3).

Elemental analysis: ***calculated*****:** $C_{13}H_{19}ClNNaO_3S$: C 47.6%; H 5.8%; N 4.3%. ***found***: C 45.9%; H 6.3%; N 4,4%.

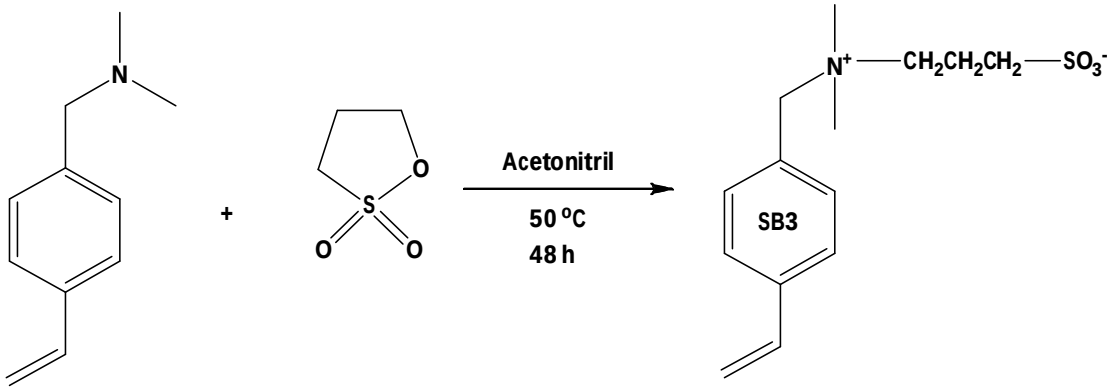
5.2.3 Preparation of the sulfobetaine monomer SB3

5.2.3.1 Synthesis of 4-vinylbenzyl-dimethylammonio propanesulfonate SB3

The synthesis of 4-vinylbenzyl-dimethylammoniopropansulfonat (SB3) follows the polymer analogous reaction route described by *Jiang and Irgum* [119] using the reaction of 4-vinylbenzyl-*N,N*-dimethyl amine with 1,3-propanesultone. In a two-neck flask under inert

atmosphere 3.34 ml 4-vinylbenzyl-*N,N*-dimethylamine (0.020 mol, 161.25 g/mol, 3.22 g, 1.0 equiv) were dissolved in 100 ml of acetonitrile and 3.40 ml of 1,3-propanesultone (0.020 mol, 122.14 g/mol, 2.44 g, 1.0 equiv) was added. In addition, a few flakes 4-tert-butylcatechol (TBC) was added to prevent possible polymerization.

The reaction mixture was heated to 223 k for 48 h. The solvent was removed under reduced pressure. The product was washed with acetonitrile several times to give 5.2 g of SB3 as colorless powders, which corresponds to a yield of 90%. The product was stored under inert gas atmosphere at 248 k.



4-vinylbenzyl-N,N-dimethylamine

Figure 5.6: Synthesis of 4-vinylbenzyl-dimethylammonio propanesulfonate SB3 [126].

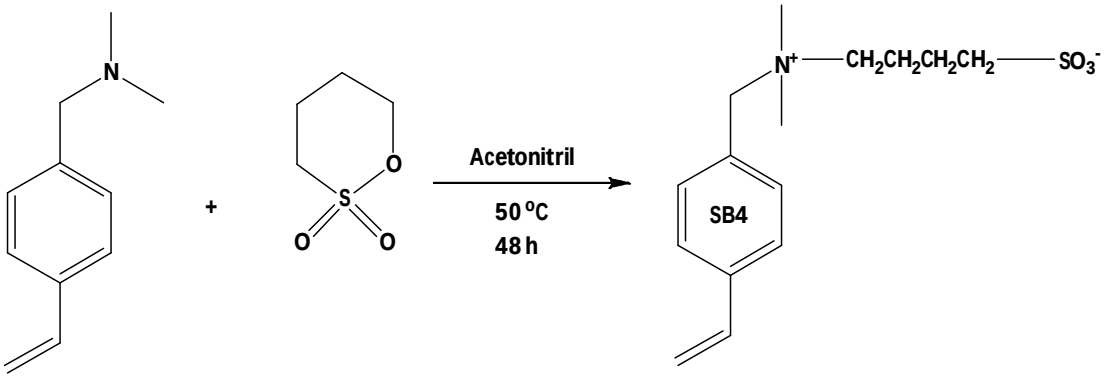
^{1}H NMR (300 MHz, D_2O): 7.55 (m, 4H, CH_{aromat}); 6.82 (dd, 1H, ^3J = 11.0 Hz and 17.7 Hz, CH_{olefin}); 5.93 (d, 1H, ^3J = 17.7 Hz, CH_{trans}); 5.40 (d, 1H, ^3J = 11.0 Hz, CH_{cis}); 4.48 (s, 2H, $CH_{2,\ benzyl}$); 3.43 (m, 2H, NCH_2); 3.03 (s, 6H, 2 × CH_3); 2.96 (t, 2H, ^3J = 7.4 Hz, SCH_2); 2.30 (m, 2H, -CH_2).

Elemental analysis: *calculated*: $C_{14}H_{21}NO_3S$: C 59.3%; H 7.5%; N 4.9%. ***found***: C 58.5%; H 7.4%; N 4.8%.

5.2.4 Preparation of the sulfobetaine monomer SB4

5.2.4.1 Synthesis of 4-vinylbenzyl-dimethylammonio butanesulfonate SB4

The preparation of 4-vinylbenzyl-dimethylammonio butanesulfonate (SB4) succeeded analogously to the preparation of the monomer SB3, only the 1,3-propanesultone was replaced by 1,4-butanesultone. In a two-neck flask under nitrogen atmosphere 3.34 ml 4-vinylbenzyl-*N,N*-dimethylamine (0.020 mol, 161.25 g/mol, 3.22 g, 1.0 equiv) were dissolved in 100 ml of acetonitrile and 3.64 ml of 1,4-butanesultone (0.020 mol, 136.17 g/mol, 2.72 g, 1.0 equiv) was added. A few flakes (TBC) were added to prevent possible polymerization. The reaction mixture was heated to 323 k for 48 h, and then the solvent was removed. The products were washed with acetonitrile to give 5.43 g of SB4 as colorless powders, which corresponds to a yield of 89%. The product was stored under inert gas atmosphere at 248 k.



4-vinylbenzyl-N,N-dimethylamine

Figure 5.7: Synthesis of 4-vinylbenzyl-dimethylammonio butanesulfonate SB4 [126].

^{1}H NMR (300 MHz, D_2O): 7.54 (m, 4H, CH_{aromat}); 6.81 (dd, 1H, ^3J = 11.0 Hz and 17.7 Hz, $CH_{ole\ f\ in}$); 5.92 (d, 1H, ^3J = 17.7 Hz, CH_{trans}); 5.39 (d, 1H, ^3J = 11.0 Hz, CH_{cis}); 4.44 (s, 2H, $CH_{2,\ benzyl}$); 3.29 (m, 2H, NCH_2); 3.00 (s, 6H, 2 × CH_3); 2.96 (t, 2H, ^3J = 7.6 Hz, SCH_2); 2.02 (m, 2H, -CH_2-); 1.78 (m, 2H, -CH_2-).

Elemental analysis: ***calculated***: $C_{15}H_{23}NO_3S$: C 60.6%; H 7.8%; N 4.7%. ***found***: C 58.5%; H 7.9%; N 4.3%.

5.3 Production of zwitterionic stationary phases

The synthesis of SB1 to SB4 precursors were shown in the previous sections. The connection of the monomers proceeds via a surface functionalization of the substrate by a grafted radical polymerization [127, 130].

5.3.1 Surface functionalization of PS-DVB-base materials

The PS/DVB materials were made by my colleague *Kristian Lungfiel* from the Seubert group [167] via a two steps synthesis using the *Ugelstad*-procedure [168]. The highly porous support materials have a degree of 55% crosslinkage and a mean particle diameter of 4.6 microns.

5.3.1.1 Preparation of the support material

In this work, the polymer particles came directly from the synthesis process and they must be initially subjected to a pore extraction before being used as a base material for zwitterionic exchangers. 94 g of the crude PS/DVB were suspended in 1000 ml toluene for 24 h under stirring heated to boiling. The material was then filtered through a Buchner funnel with a black band filter. The solid was resuspended in 1000 ml of toluene and for additional 24 h stirred and heated to boiling. After repeated filtration, the polymer was suspended in 1000 ml 1,2–Dichloro propane and heated for 24 h to boiling. After filtration through a black band filter, the PS/DVB was dried at 338K to constant weight.

After drying the polymer was suspended in portions with a total of 2000 ml of ethanol and initially screened through a sieve with a pore diameter of 75 microns. Any existing coarse-grained residue that has not passed the sieve was discarded. Subsequently, a second screening step was carried out through a sieve with a pore diameter of 21 microns. Again, the coarse-grained non-filterable residue was discarded. The remaining material was filtered

through a black band filter and re-dried at 338 k to constant weight. The prepared material can be stored until use for the graft polymerization in sealed polyethylene containers.

5.3.1.2 Implementation of the graft polymerizations

The graft polymerization was carried out according to the route developed by *Raskop et al.* [127, 130] (EVOIII). In contrast to the procedure described by *Raskop* (standard EVO), the solvent was changed to acetone instead of ethanol. The amounts and conditions were summarized in Table 5.4 for 3 g approaches.

Table 5.4: Conditions in the EVO-synthesis of zwitterionic phases.

Parameter	per 3 g EVO approaches
Solvent	100 ml water/acetone (80:20)
Polymer sample weight	3.0 g (4 mm columns)
Monomer amount	SB1: 2.79 to 7.50 mmol SB2: 3.71 to 11.12 mmol SB3: 1.26 to 16.38 mmol SB4: 2.49 to 9.96 mmol
Radical initiator amount	1.1 equiv
Temperature	333 K
Reaction time	4 h
Radical initiator	$K_2S_2O_8$
Addition of radical initiator	15 min after addition of monomer

In a double-walled, fluid-tempered reaction vessel with 150 ml internal volume, 80 ml of water were initially introduced at 333 K under an inert atmosphere. Then, a slurry of 3.0 g PS/DVB in 20 ml acetone was added and the mixture stirred for 20 min at 5000 rpm/min.

Thereafter, the required amount of zwitterionic monomer (1.0 equiv) was added via a funnel as a solid and stirred for a further 15 minutes. After this time, 1.1-equiv radical initiator ($K_2S_2O_8$) added in solid form and the stirring speed was at 4000 rpm/min. The reaction mixture was stirred for 4 h and then added to 400 ml of ice-cold Millipore to stop reaction.

This suspension was filtered through a Büchner funnel with a black band filter and the polymer was washed with 1000 ml of water. Subsequently, the polymers were washed with 500 ml of aqueous sodium acetate solution (100 mmol/l, pH 4.75) and final washed with acetonitrile 300 ml. The final exchanger material was suspended in 20 ml of sodium acetate (100 mmol/l, pH 4.75) and can be used to pack the columns at room temperature. The slurry was in sealable glass containers.

5.4 Packing of columns

The packing of the columns with the sulfobetaine materials was carried out by the down-fill technique [43]. For packing a column of the dimension 4 mm i.d. × 100 mm length, 10 ml of the polymer suspension were treated with ultrasonic for 5 min. This corresponds to a solid content of 1.5 g dry matter. The packing apparatus was shown in Figure 8.5. The apparatus was assembled together according to the sketch and rinsed with about 100 ml of ultrapure water. The bottom of the post column was fitted with a steel frit to keep the packing material in the column body.

The reservoir was emptied and then refilled with the suspended zwitterionic exchanger material from the upper edge via a syringe. To avoid air in the system, it is necessary to fill the upper edge of the reservoir with water. For fast packing, a pressure of 500 bar was applied. Acetate buffer (100 mmol/l, pH 4.75) was used as packing fluid for all functionalized materials. The pressure was kept constant as long as required to pump 200 ml pack fluid through the column. The pressure was released slowly and the column body was separated from the pre- and postcolumns and sealed.

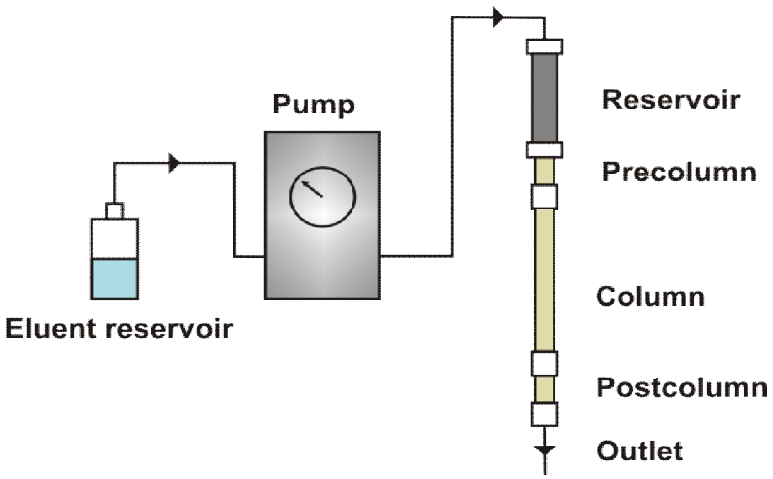


Figure 5.8: Schematic structure of the column packing equipment.

5.5 Capacity requirements

The capacities of the zwitterionic exchangers were varied using different amounts (1.26 to 16.38 mmol) of the sulfobetaine monomers compared the procedure described in reference [10]. For this work, the capacity of the functionalized exchanger materials must be determined. In this work, the capacities of the four-sulfobetaine exchangers were determined by two methods. As a first method, the capacities were determined using the sulfur contents measured by x-ray fluorescence on the sulfur $K\alpha_{1,2}$ line at 5.3731 Å. The second method was a combustion elemental analysis. Elemental analysis was conducted for all synthesised exchangers to determine the sulfur and nitrogen contents.

5.5.1 Capacity determination by X-Ray fluorescence analysis

In this method, 250 mg of dried polymer (PS/DVB) samples were applied to the sample holder. Reference standards were prepared using unfunctionalized polymers. Portions of 1.0 g of unfunctionalized polymer were first suspended in 20 ml of acetone/water (20:80). To these suspensions, different volumes of 100 µl to 20 ml of ammonium sulfate solution (100

mmol/l) were added to obtain reference standards with 10-2000 µmol sulfur per gram of polymer. After thorough mixing, the suspensions were dried at 338 K. The crystalline ammonium sulfate is randomly distributed over the unfunctionalized PS/DVB. Using the reference standards, a calibration curve was established and used for the determination of the sulfur contents of the zwitterionic polymers.

5.5.2 Capacity determination by elemental analysis

The second method for determining the total capacity was combustion elemental analysis. This allows the determination of the sulfur and the nitrogen content of the polymers prepared. It can thus be determined independently of the elemental analysis of both functional groups sulfobetaine exchangers. For this, 10 mg of the dried polymer were combusted and the content of nitrogen as well as the content of sulfur was determined.

5.6 Zwitterionic stationary phases characterization

5.6.1 Surface excess isotherms and thermodynamic void volume determination using a minor disturbance method

The void volumes of sulfobetaine stationary phases were determined using a minor disturbance method. The minor disturbance peaks were recorded using water/acetonitrile mixtures. The SB_n-column was equilibrated with mobile phases containing 0%, 2%, 5%, 8%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 92%, 95%, 98%, 99% and 100% volume fractions of acetonitrile by pumping at least 30 ml of the solvent mixture (The flow of the mobile phase was 0.5 ml/min). After each equilibrium was reached, 5 µl of solvent mixture was injected. The thermodynamic void volume V_0 (Equation 5.1) of the columns was obtained by integration (from 0% to 100% of the solvent) of the minor disturbance peak retention volumes over the eluent phase composition range [162]:

$$V_0 = \frac{1}{C_{ACN,H_2O}} \int_0^{C_{ACN,max}} V_R \left(c_{ACN,H_2O}\right) dc_{ACN,H_2O} \tag{5.1}$$

Here, V_R is a retention volume of the minor disturbance peak, $C_{ACN,\ H2O}$ is the concentration of the solvent [mol/l]. The excess adsorption isotherms ($\Gamma_{ACN,H20}$) of organic modifiers from the water on covalently bonded stationary phases were studied, using several different sulfobetaine columns. Excess adsorption isotherms were calculated using the following Equation 5.2 [154]:

$$\Gamma_{ACN,H_2O}(C_{ACN,H_2O}) = \frac{1}{S}\int_0^{C_{ACN,H_2O}} \left(V_R\left(c_{ACN,H_2O}\right) - V_0\right) dc_{ACN,H_2O} \qquad (5.2)$$

Here, S is a total surface area of the adsorbent in the column.

5.7 Preparation of the measurement solutions

The chemicals used to prepare the solutions were already summarized in Tables 5.2 to 5.3. All reagent solutions were prepared using distilled deionised Milli-Q water (Millipore, Bedford, MA, USA) having a conductivity of 18.2 MΩ.

5.7.1 Standard solutions for ZIC-mode

As part of the chromatographic measurements in various modes (ZIC, ZIC-HILIC), a variety of measurement standards were needed. The procedure for the preparation of these standards in all cases was the same. The anion stock solutions were prepared in 100 ml HDPE-containers with concentrations of 2000 mg/kg. All standards were prepared by dilution from stock solutions. The concentration of 2000 mg/kg refers to the concentration of the pure anions. These stock solutions were stable at room temperature. The measurement solutions were obtained by dilution with ultrapure water and can be used for weeks.

5.7.2 Standard solutions for ZIC-HILIC mode

The pharmaceuticals stock solutions of Deferoxamine mesylate (DFOM), Thiamine hydrochloride (B1), Diclofenac sodium (DCS), Cyclopentolate hydrochloride (CPH), Dexame

thasone sodium phosphate (DMSP), Tetracaine hydrochloride (TCH), Pilocarpine hydrochloride (PCH) were made by dissolving the pure compounds in ultrapure water, Chloramphenicol (CPA) was dissolved in 100% acetonitrile with concentrations of 1000 mg/kg and stored at 277K. The measurement standards were prepared by dilution with water or acetonitrile and stored at 277 k. They can be used over several weeks. Iron and aluminum stock solutions were prepared by dissolving ferric nitrate nonahydrate and aluminum nitrate nonahydrate in water. The standards were prepared by dilution with water and then using these solutions to form chelating complexes with deferoxamine mesylate.

5.7.3 Eluents

Eluents for each operating modes ZIC and ZIC-HILIC were using different compositions. Eluents were prepared by dissolving pure substances in ultrapure water. For ZIC-mode, sodium acetate was used as eluent, whilst the eluent used in ZIC-HILC mode was a mixture of sodium acetate and acetonitrile. Eluents for ZIC-HILC mode using organic modifiers were made using a high-pressure gradient system with two HPLC pumps. The mobile phase should preferably be degassed daily to avoid pump related interferences and to lower detector noise.

5.8 Chromatography systems

In their construction, all chromatographic systems follow the detection principle and the eluent mixture. In the following chapter, the apparatuses used were classified according to detection methods and illustrated with sketches of the corresponding system.

5.8.1 Setup of the IC–System with UV/VIS-detection

The system for measurements with UV/VIS detection uses a column thermostat and two HPLC pumps. The use of two pumps allows the mixing of aqueous phase and organic modifier in the high-pressure range. The pump for pumping the aqueous phase was made of PEEK, while for the organic component was a steel pump head was used. The schematic structure was shown in Figure 5.9. The eluents were degassed before passing through the pump using

an on-line vacuum degasser. Afterwards, both aqueous phase and organic modifier pass individually to a T-shaped connection piece. A stainless steel capillary loop was used to ensure a homogeneous mixing of the eluent. We used a pulsation dampener between the pump and the injection valve to protect the column material against pressure drops caused by the injector. In this way, fast gradient separations as well as fast eluent variations were possible without prior mixing of the components. This system was used to separate the anions in ZIC and ZIC-HILIC modes, as well as the separation of pharmaceutical compounds in ZIC-HILIC mode and for the measurement of the excess isotherms of sulfobetaine exchangers via the minor disturbance method.

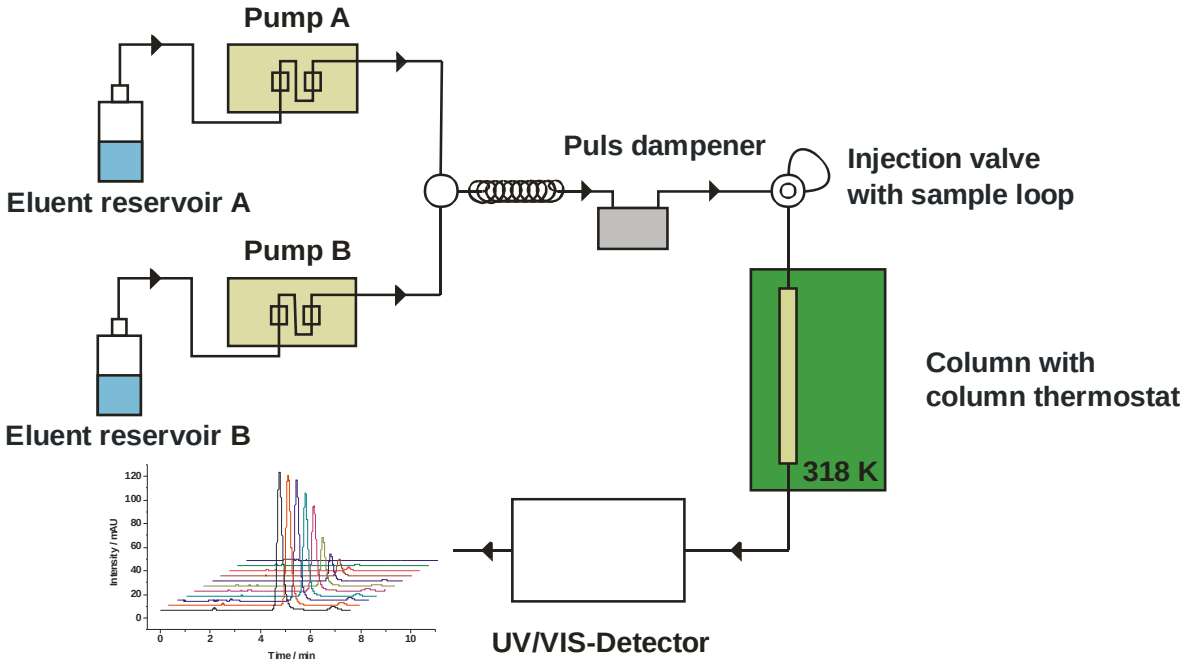


Figure 5.9: Schematic of the IC-system with UV/VIS detection.

5.8.2 Setup of the IC-system with ICP-AES detection

The IC system coupled on-line with ICP-AES detection also uses a column thermostat and HPLC pump. The aqueous eluent phase and the organic modifier were mixed (80:20) before they passed to the pump. The 20% of the organic modifier was used in all measurements in order to avoid extinguishment of the plasma. The pump for pumping the aqueous phase was made of PEEK. The schematic structure was shown in Figure 5.10. The eluent passed to a T-shaped connection, which was linked with a peristaltic pump on-line to add water in order to dilute the mobile phase. We used a pulsation dampener between the pump and the injection valve. This system was used to separate the metal–Deferoxamine complexes in ZIC-HILIC modes.

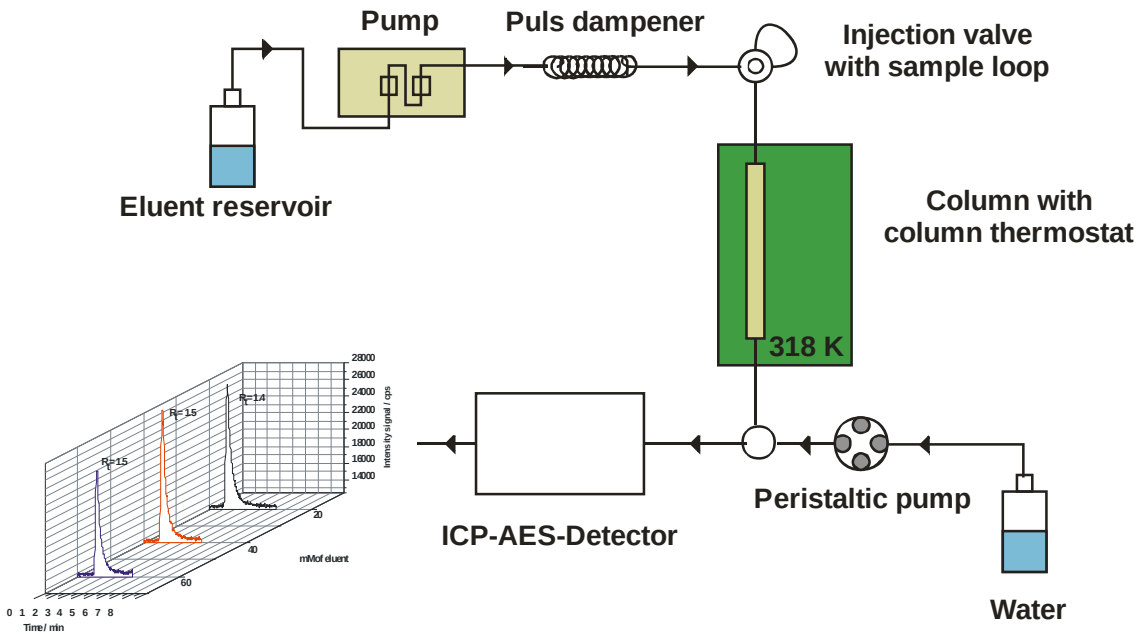


Figure 5.10: Schematic of the IC-system with ICP-AES detection.

5.9 Instrument specifications

5.9.1 Specifications of the chromatographic systems

The experimental work was performed with a modular IC System (Metrohm AG, Herisau, Switzerland). The specifications of the systems were summarized in Table 5.5.

Table 5.5: Instrument specifications of the IC systems

Device	Specification	Manufacturer
Pump	709 IC pump (PEEK head)	Metrohm
	709 IC pump (Steel head)	Metrohm
	844 UV/VIS Compact IC	Metrohm
	790 Personal IC	Metrohm
Degasser	ERC-3315	Gynkotek
Interface	762 IC Interface	Metrohm
Autosampler	766 IC Sample Processor	Metrohm
Injection valves	820 IC Separation Center	Metrohm
	844 UV/VIS Compact IC	Metrohm
	790 Personal IC	Metrohm
Detectors	Variable wavelength monitor 87.00	KNAUER
	844 UV/VIS Compact IC	Metrohm
	ICP-MS 7500ce	
Data analysis	Software ICNet 2.3 SR 3	Metrohm
	790 PC Software 1.0	Metrohm

5.9.2 Specifications of other instrument

In addition to the chromatographic systems, more analytical instruments have been used to characterize zwitterionic monomers and produced sulfobetaine exchangers. The instrument specifications were summarized in Table 5.6.

Table 5.6: Instrument specifications of the other devices.

Device	Specification	Manufacturer
Elemental analysis	Vario Micro cube	Elementar
Hydraulic unit (packPump)	LP-1078	Haskel
X-Ray fluorescence	ARL Optim'X Software Oxsas 1.2	Thermo Fisher
ICP-AES	Spectroflame P Software Smart Analyzer 2.25	Spectro Analytical Instruments GmbH

6 Summary and outlook

The influence of capacity on separation characteristics has been investigated for a series of zwitterionic exchangers prepared by free radical graft polymerization of PS/DVB particles. Moreover, zwitterionic exchangers with different intercharge chain length (SB1, SB2, SB3 and SB4) have implemented in this study. The important aspect in our study is the monitoring of the influence of altered capacities of the zwitterionic stationary phases in ZIC and ZIC-HILIC mode. The zwitterionic stationary phases exhibit a similar behavior under ZIC and ZIC-HILIC conditions. Furthermore, increasing capacity of the sulfobetaine exchangers lead to various interactions of analyte anions with cation sites on sulfobetaine exchangers. These various interactions are stemmed from the different flexibility of the sulfobetaine chains influencing their ability to form intra- and intermolecular ion pairs. Thus, the spacer length between the charges and exchange capacity of ZIC-exchangers exhibit an influence on the retention of analyte anions.

The capacity of exchangers plays a significant role in the separation mechanism of anions. In the ZIC mode, the SB2-exchanger exhibits a different separation mechanism in comparison to a previous study [10]. In one hand, the low-capacity SB2-exchanger (130 $\mu eq\ g^{-1}$) in the previous study showed constant retention for anions by varying eluent concentrations. On the other hand, the higher capacity SB2-exchangers (300-790 $\mu eq\ g^{-1}$) in this work exhibits decreasing retention for anions with increasing eluent concentration. Therefore, the separation mechanism of anions using SB2-exchanger follows standard ion exchange mechanism. Eventually, after the investigation the elution system, the variation of exchange capacity does not lead to a significant change on the separation selectivity and efficiency for anions.

HILIC-retention induced by partitioning still lacks a comprehensive theoretical explanation. The investigation of a series of sulfobetaine-exchangers gives more insight into the mecha-

nism of separation in ZIC-HILIC mode. SB1, SB3 and SB4 exchangers seem to behave as expected for ZIC-HILIC materials. In contrast, the SB2-exchangers show RP-loke behavior. The different behaviors of SB_n-columns may occur due to the difference in the geometric alignment of sulfobetaine molecules. Interestingly, the SB3-exchanger shows some selectivity changes in the elution sequence of anions under ZIC-HILIC conditions when the effect of acetonitrile content was studied. This change is attributed to the superposition of the chaotropic, hydrophilic interactions and the hydration enthalpy of anions.

Remarkably, it has been observed for SB4-exchanger under ZIC-HILIC conditions at low buffer strength, that selectivity changes occurred caused by the limited semi-immobilized aqueous adsorbed layer on this type of zwitterionic stationary phases. This retention behavior of anions could be caused only via electrostatic interaction. Eventually, the final separation mechanism is most probably a superposition of hydrophilic and electrostatic interactions to the sulfobetaine stationary phase. Otherwise, no changes were observed in the selectivity order of anions in ZIC-mode.

The current work revealed the differences of the retention characteristics of eight pharmaceutical compounds on four new sulfobetaine stationary phases in HILIC separation using a unique column test set with the sulfobetaines and pure anion or cation exchangers based on the same core material and using the same functionalization strategy. The retention characteristics are based on RP, ZIC-HILIC and ion exchange resulting ion a mixed mode for the tested pharmaceuticals. The reason for this behavior (HILIC and RP) can be attributed to the hydrophilicity of the pharmaceuticals, when the variation of acetonitrile content was studied. Furthermore, the separation of the pharmaceuticals relied on ion exchange interactions with the stationary phase as shown during the variation of the ionic strength of the eluent. ZIC-HILIC stationary phases are suitable for the determination of the hydrophobicity characteristics of six investigated compounds which showed hydrophobic behavior.

Linear correlations were obtained between the logarithm retention factor and the concentration of acetonitrile in the mobile phase with a $R^2 > 0.94$. Geometrical alignment of these zwitterionic materials plays a significant role in the relationship between $\log k'_w$ and $\log P$ and shows excellent agreement between the experimental and the calculated values.

These set of stationary phases are a new tool which can be used to investigate the retention mechanisms of multifunctional compounds such as pharmaceuticals. Moreover, it can help to choose the proper stationary for complex analytical problems encountered in pharmaceutical, biological and environmental samples. The PS/DVB used as core material can play a key role in the retention of these pharmaceuticals in comparison to silica or methacrylate core materials. The interesting findings on the separation mode of non-polar compounds and the separation mechanism of eight pharmaceutical compounds are a motivation for further investigations on new pharmaceuticals to increase knowledge about the ZIC-HILIC separation mechanism and to create optimized separation applications.

Zwitterionic ion chromatography on-line coupled to ICP-AES is an appropriate tool for the investigation of metal binding of trihydroxamic acid (DFOM). Deferoxamine is a strong chelator for multiple charged ions such as Fe(III) and Al(III), which are important ions from a biological point of view. It could be demonstrated that metal-DFOM separations on sulfobetaine exchangers are mainly driven by a cation exchange mechanism**.** In spite of numerous publications in the literature which confirm that zwitterionic type materials are usually well suited for anion separation, no investigation had been carried out to separate cationic organic compounds. The current study confirmed the ability of ZIC-HILIC columns to separate metal-complexes, organic cations and non-polar compounds. Nevertheless, more experiments are needed to extend the mechanistic knowledge in ZIC-HILIC mode for such compounds.

The change in eluent composition and the presence of polar and non-polar functional groups in the structure of sulfobetaine exchangers lead to changes in the excess adsorption of water from acetonitrile-rich mobile phases. It was found that the sulfobetaine exchangers adsorb water strongly when the concentration of water is low in binary aqueous organic mixtures. Interestingly, the spacer length between the charged functional groups and also capacity of zwitterionic exchangers play an important role in the water layer enrichment at the sulfobetaine surface. Additionally, it was observed that increasing chain length between charges of sulfobetaines increases the excess water adsorption, too.

Notwithstanding the fact that the increased capacity-exchangers lead to increased polar functional groups on the surface, consequently, the water adsorption from acetonitrile-rich

eluent increases at column surface. Nevertheless, increasing the capacity of the zwitterionic columns resulted in decreased adsorption of water on sulfobetaine exchangers. This is due to the decreased free anion and cation sites available on the zwitterionic exchangers. It is remarkable that all sulfobetaine type exchangers under study exhibit a similar behavior when investigated with regard to the excess adsorption isotherms of water. The correlation between the retention factors of eight pharmaceutical solutes and the maximum excess of water adsorption on the series of zwitterionic column is not powerful, due to the different interaction of the solutes with the zwitterionic exchangers. In conclusion, it is possible that the excess adsorption isotherms of water onto zwitterionic exchangers are an useful tool for providing information on HILIC properties of zwitterionic columns.

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Curriculum Vitae

Name: Ashraf Saad Rasheed Al-Ayash

Date of birth: 23.12.1978

Place of birth: Baghdad-Iraq

Marital status: Married

E-mail: ashraf_analytical@yahoo.com

rasheed@students.uni-marburg.de

Academic degrees:

1. **M.Sc.** Analytical Chemistry, December 2003, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq.
   Thesis Title: "AA Spectrometric – Vis Estimation of Drug Desferrioxamine in Desferal Using Gold and Vanadium as Mediating Metals ".
2. **B.Sc.** Chemistry, July 2000, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq.

Experience:

1. **October. 2010 to Present:** PhD student in the Faculty of Chemistry, Philipps-Universität Marburg, Germany.
2. **January. 2009 to Present:** Lecturer of Analytical Chemistry, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq.
3. **May. 2005 to 2008:** Assistant Lecturer of Analytical Chemistry, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq.
4. **January.2004 to 2006:** Formulation of drug and Director of laboratories, Al – Shafaa drug Factory, Baghdad, Iraq.

Publications:

[1] A. Q. Waleed, Z. A. Khammas, A. S. Al-Ayash, and F. Jasim, "An indirect atomic absorption spectrophotometric determination of trifluoperazine hydrochloride in pharmaceuticals", *Arabian Journal for Science and Engineering,* vol. 36, pp. 553-563, 2011.

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Conferences:

1. The 24th International Ion Chromatography Symposium-IICS, Berlin, Germany, September 17-20, 2012 [Poster Presentation and Oral Presentation].
2. The 3th International Natural Sciences Conference, March 24-26, 2009, College of Science, University of Baghdad, Baghdad, Iraq. [Oral Presentation].

3. The 3th International Conference on Chemistry, January 18-19, 2002, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq. [Oral Presentation].
4. The 3th International Conference on Chemistry, January 18-19, 2002, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq. [Oral Presentation].

Oral presentation:

1. Oral presentation on my PhD work in the weekly seminar of the research groups of analytical chemistry in the Faculty of Chemistry, University of Marburg, June 18, 2013, Marburg, Germany." Influence of capacity on the retention characteristics of inorganic ions in zwitter ion chromatography and hydrophilic interaction chromatography on four different sulfobetaine stationary phases".
2. Ashraf S. Al-Ayash; Andreas Seubert:"Retention behavior of pharmaceuticals on new polymer based ZIC-HILIC stationary phases in comparison with almost identically constructed anion and cation exchangers", in the conference 24th International Ion Chromatography Symposium-IICS" Berlin, Germany, September 2012.
3. Ashraf.S. AL-Ayash, Yasmeen H. Muhamad, Sahel A.Ghafouri:"Spectrophotometric determination of epinephrine in pharmaceutical preparations using praseodymium as mediating metals", In the 3th International Natural Sciences Conference, March 24-26, 2009, College of Science, University of Baghdad, Baghdad, Iraq.
4. Ashraf.S. Al-Ayash, Zuhair A-A.Khammas and Fadhil Jasim:" Indirect electrothermal atomization atomic absorption spectrometric determination of the drug desferrioxamine in some pharmaceutical preparations using vanadium (V) as a mediating element", in the 3th International Conference on Chemistry, January 18-19, 2002, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq.
5. Oral presentation on my M.Sc. work in 15 September 2003, Department of chemistry, College of Science, University of Baghdad, Baghdad, Iraq."AA Spectrometric – Vis estimation of drug desferrioxamine in desferal using gold and vanadium as mediating metals".

Membership:

1. Member in the German Chemical Society (GDCh).
2. Member in the International Separation Science Society-USA (CASSS).
3. Member in the Iraq Chemical Society.

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2. Full Scholarship (October 2010 -present) DAAD (German Academic Exchange Service).

References:

1. **Prof. Dr. Andreas Seubert**
   University of Marburg
   Faculty of Chemistry
   Hans-Meerwein-Straße
   35032 Marburg, Germany.
   E-mail: seubert@staff.uni-marburg.de

2. **Prof. Dr. Zuhair A-A Khammas**
   Department of Chemistry
   College of Science for Women
   University of Baghdad
   Jadiyria – Baghdad, Iraq
   E-mail : dr_zuhair52@yahoo.com

3. **Prof. Dr. Muthana Shanshal**
   (Prof.Dr.rer.nat.habil)
   Department of Chemistry
   College of science
   University of Baghdad
   Jadiyria – Baghdad, Iraq
   E-mail: mshanshal2003@yahoo.com

Erklärung

Ich versichere, dass ich die vorliegende Dissertation mit dem Titel

Influence of capacity on the retention characteristics in Zwitter Ion Chromatography (ZIC) and ZIC-Hydrophilic Interaction Chromatography (HILIC) on four different sulfobetaine stationary phases

selbständig, ohne unerlaubte Hilfe angefertigt und mich dabei keiner anderen als der von mir ausdrücklich bezeichneten Quellen und Hilfen bedient habe. Die Dissertation wurde in der jetzigen oder einer ähnlichen Form noch bei keiner anderen Hochschule eingereicht und hat noch keinen sonstigen Prüfungszwecken gedient.

Ort, Datum

Ashraf Saad Rasheed