



## Separation of inorganic anions using a series of sulfobetaine exchangers

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### ABSTRACT

A set of five new sulfobetaine exchangers with inner quaternary amines and outer sulfonic acids have been prepared. A series of zwitterionic precursors was attached to highly porous divinylbenzene polymer using a grafting reaction, which allows a flexible adjustment of the degree of functionalisation. The resulting materials have identical spacers to the polymeric backbone and differ only in chain length between the charged functional groups. Capacities of the stationary phases were analysed by two different methods based on elemental analysis and the results obtained were found to correlate. The application of combustion elemental analysis proved an identical molar content of sulfur and nitrogen. The distance between the charged functional groups is varied from one to five methylene groups for a better understanding of the retention behaviour of inorganic anions on zwitterionic stationary phases. Inorganic anions were separated using sodium acetate eluents with varying ionic strength and pH and the behaviour of all columns has been compared to each other and to ZIC-HILIC and ZIC-pHILIC columns from Merck SeQuant. The exchangers with two and five methylene groups between the charges showed the highest retention factors. Polarity and accessibility of the anion exchange sites are dependent on the distance between the charged groups and the flexibility of the chains. These properties have a strong influence on anion separations. The exchanger with two methylene groups between the charged functional groups showed the biggest difference as compared to the commercially available ZIC-HILIC and ZIC-pHILIC exchangers.

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### 1. Introduction

During the last ten years hydrophilic interaction chromatography (HILIC) gains growing interest in research and literature [1,2]. The main reason for this interest can be seen in the uncommon chromatographic conditions concerning polarity of the mobile and the stationary phase that can be used in HILIC separations.

In general HILIC is a chromatographic method in which separation of analytes is achieved by partitioning between a water rich, virtually immobilised layer on a hydrophilic stationary phase and a rather hydrophobic mobile phase containing organic modifiers at percentages of 60 and more [3]. The polarities of the mobile and stationary phases therefore are similar to those in normal phase HPLC (NP-HPLC) [4] without having to deal with the disadvantages of this technique like the necessity of water free solvents to ensure reproducibility. In many cases the elution order in HILIC is exactly inverted compared to the elution order in RP-HPLC [5] and is specially suited for the separation of polar organic substances, which elute in or near the hold-up volume in RP-HPLC. Among this group of analytes are carbohydrates [6,7], amino acids [8], peptides [9] as well as miscellaneous pharmacological agents [10]. A large number

of physiologically important substances can be separated using this technique which has contributed to its popularity.

In HILIC, there is a large variety of materials used as stationary phases. Differences can be found in the utilised functional groups as well as in the core materials. Beside classical exchangers like diol- or amido-materials, zwitterionic materials have been in HILIC separations for a few years now. Zwitterionic exchangers give an additional advantage beyond the polarities of the phase. They carry ionic groups which can influence the chromatographic process by providing the possibility of electrostatic interactions with charged analytes. Therefore they can be utilised detached from the HILIC mechanism using completely aqueous eluents. This process has been called zwitterionic ion chromatography (ZIC) by Cook [11,12]. When zwitterionic materials are used for HILIC separations, the method can be described as ZIC-HILIC.

The first exchanger carrying both quaternary amine and sulfonic acid functionality was developed in 1951 by Stach [13]. More recently, zwitterionic stationary phases were prepared by covalent attachment of  $\alpha$ -amino acids to silica gel [14,15]. In addition, a large number of studies have been carried out using C18-materials dynamically coated with zwitterionic tensides [16,17]. Although widely applied, these materials suffer some disadvantages. Amino acid exchangers show a strong dependency of their charge with changing eluent pH [18] while dynamically coated materials suffer from bleeding tensides. In 1986 Hartwick was able to develop the first material in which zwitterionic sulfobetaines were cova-

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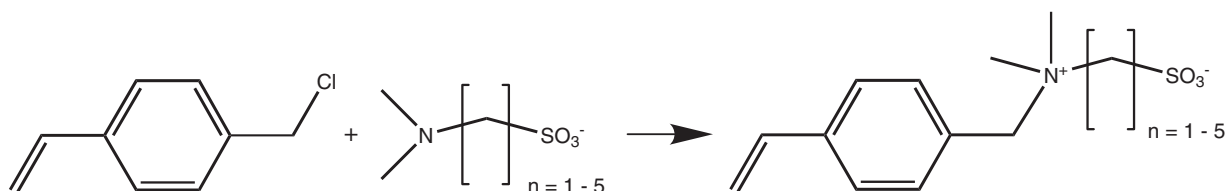


Fig. 1. Synthesis of the zwitterionic precursors.

lently bond to a silica core [19]. Further improvements were made in the 1990's from which the nowadays commercially available sulfobetaine-type materials ZIC-HILIC and ZIC-pHILIC from Merck SeQuant derived [20].

These covalently bond stationary phases allowed the application of ZIC-HILIC materials in routine analysis. However the synthesis of such covalently bond exchangers having zwitterionic groups attached to silica or polymeric cores still is a challenging task [18]. Hence, scientific progress in the field of ZIC and ZIC-HILIC is mainly limited to application development using these existing commercial exchangers. Despite the increased number of applications of these zwitterionic stationary phases, there still is much work to be done in the study of the mechanisms involved and in the improved understanding of the principles governing the separation in ZIC and ZIC-HILIC.

To reduce complexity of the separation mechanisms, it is useful to study ZIC separations in completely aqueous environments without regarding the influence of hydrophilic partitioning. ZIC plays an important role in the characterisation of zwitterionic materials. Utilising dynamically coated C18-materials Hu et al. carried out a large number of experiments on this field [21,22]. These studies led to the proposed mechanism of ZIC [22].

When investigating the principles governing a separation, certain factors have the potential to influence the retention and separation. One such parameter is the core material used. In ZIC silica and polymeric phases as well as dynamically coated C18-materials are currently used. While silica gel provides a very high stability versus organic modifiers, polymers like PS/DVB are more stable concerning eluent pH. Dynamically coated C18-materials can easily be prepared and offer the ability to perform ZIC separations but suffer from bleeding and cannot be used for ZIC-HILIC. In sulfobetaine based materials, having both quaternary amine and sulfonic acid functionality, the distance between the amine and the sulfonic acid is expected to influence retention. The effect of this distance has been previously studied using dynamically coated C18-materials [23]. For coating tensides having three respectively ten methylene groups between the charged functional groups were prepared and compared under ZIC conditions but no significant difference in behaviour was found. The goal of our work is to determine the chromatographic properties of covalently bond sulfobetaine exchangers with a more narrow range of interchange chain lengths.

For short distances from one up to five methylene groups, it can be expected that the polarities of the materials as well as their ability to saturate the charges via inter- and intramolecular interactions will show the strongest variation. To determine this, five new zwitterionic materials having one to five methylene groups between the amine and the sulfonic acid, were synthesised and studied.

The chromatographic properties of this series of zwitterionic sulfobetaine exchangers was evaluated and compared to those of commercially available ZIC-HILIC and ZIC-pHILIC phases. While the prepared zwitterionic exchangers are based on PS/DVB, ZIC-HILIC and ZIC-pHILIC have both silica and polymeric cores. To be able to determine the influence of the distance between the charged groups, it is extremely important to be able to prepare zwitterionic exchangers having identical core material, identical spacers to the

polymeric backbone as well as comparable capacities, so the interchange chain length is the only difference between the materials to be compared.

## 2. Experimental

### 2.1. Reagents and solutions

Barium bromide (95%) was purchased from ABCR (Karlsruhe, Germany). Acetic acid (100%) was purchased from Carl Roth (Karlsruhe, Germany). Sodium bromide ( $\geq 99.0\%$ ), sodium hydroxide (50%) and sodium thiocyanate ( $\geq 98\%$ ) were obtained from Fluka (Buchs, Switzerland). Sodium acetate ( $\geq 99\%$ ) and sodium hydrogen phosphate monohydrate ( $\geq 99\%$ ) were purchased from J.T. Baker (Deventer, The Netherlands). Ammonium hydroxide (25%), sodium fluoride ( $\geq 99.5\%$ ), sodium iodide ( $\geq 99.5\%$ ), sodium nitrate ( $\geq 99.5\%$ ), sodium nitrite ( $\geq 99\%$ ) and sodium sulfate ( $\geq 99\%$ ) were from Merck (Darmstadt, Germany). Sodium hydrogen carbonate ( $\geq 99.7\%$ ) as well as sodium chloride ( $\geq 99.8\%$ ) were purchased from Riedel-de Haën (Seelze, Germany). Sodium nitrate ( $\geq 99.0\%$ ) was obtained from Sigma-Aldrich (Seelze, Germany). Ultrapure water having a conductivity of  $18.2 \text{ M}\Omega$  was prepared using a Milli-Q water purification system (Millipore, Bedford, MA, USA). Eluents and stock solutions of sample ions were prepared by dissolving the salts directly in ultrapure water. Reagents for the synthesis of the zwitterionic precursors were used in highest available purity [24]. The core material consisted of highly crosslinked macroporous polystyrene/divinylbenzene copolymer. The crosslinking degree was 55%, particle size was  $4.6 \mu\text{m}$ . ZIC-HILIC and ZIC-pHILIC columns were obtained from Merck SeQuant (column dimension:  $100 \text{ mm} \times 4.6 \text{ mm I.D.}$ ,  $5 \mu\text{m}$  particle size).

### 2.2. Synthesis of zwitterionic precursors

The preparation of covalently bond zwitterionic exchangers is based on the synthesis of the corresponding zwitterionic precursors [24]. They provide on one hand the zwitterionic groups with the expected distances between the charged groups and on the other hand the functionality for the attachment to the polymeric resin by a grafting reaction.

Therefore zwitterionic precursors are prepared as aromatic monomers by simple nucleophilic substitution reactions between a monomeric styrene derivative as a spacer and tertiary amines. These tertiary amines with one to five methylene groups between the amine and the sulfonic acid are prepared via simple organic reactions. Afterwards they are attached to the spacer. In doing so, the substitution of a chloride atom gives the quaternary amines as shown in Fig. 1.

### 2.3. Characterisation of the precursors via RP-HPLC

These precursors have the great advantage of being monomeric and therefore analysable by molecule methods like NMR-spectroscopy and RP-HPLC using UV detection. For HPLC separations a standard HPLC system (Hewlett Packard 1100 series, Agilent, Santa Clara, CA, USA) was used. The separations of the precursors

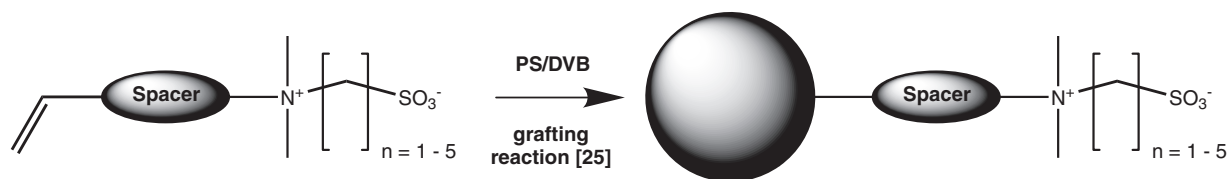


Fig. 2. Scheme of the grafting reaction leading to zwitterionic exchangers.

sors shown in Fig. 1 are carried out under isocratic conditions on a Nucleosil C18 column (250 mm × 4 mm I.D., 7 μm particle size, Macherey Nagel, Düren, Germany). The eluent consists of methanol/water/phosphate buffer (20 mM, pH 3) at percentages of 30:50:20. Detection is performed at 254 nm. The concentration of the analytes is about 10 mg/l using a 20 μl injection loop.

#### 2.4. Functionalisation of polymeric particles by grafting

Functionalisation of the resin is performed by a grafting reaction following a preparation invented by Raskop et al. [25]. By doing so, the zwitterionic precursors are covalently bonded to the polymeric resin. The stationary phases are packed using PEEK columns (100 mm × 4 mm I.D.).

#### 2.5. Determination of capacities

Determination of the capacities of zwitterionic exchangers is a challenging task. All known methods of determining capacities dynamically in chromatography cannot be applied. Therefore, two non-dynamical methods were tested. For both methods the material was first washed with 500 ml (per 3.0 g of the resin) of 100 mM aqueous sodium acetate solution (pH 4.75) followed by 500 ml of acetonitrile.

The first method is combustion elemental analysis (vario EL, Elementar, Hanau, Germany). For this, 40 mg of the dried polymer are combusted and the content of nitrogen as well as the content of sulfur are determined. The nitrogen content is determined by high temperature combustion, separation and determination via thermal conductivity detector. The sulfur content is determined by oxidative decomposition and photometric titration of sulfate with barium perchlorate.

The second method is the determination of sulfur via X-ray fluorescence (XRF) (ARL Optim'X, Thermo Fisher, Waltham, MA, USA). 250 mg of the dried polymers are put into sampling cups and detected directly by XRF on the sulfur K $\alpha_{1,2}$  line at 5.3731 Å using the PET crystal.

#### 2.6. Chromatographic conditions

All chromatographic separations – except the ones described before for the characterisation of the zwitterionic precursors – are carried out using a Modular IC System (Metrohm AG, Herisau, Switzerland). For pumping the eluent a 709 IC pump is used. The injection valve (5 μl injection loop) as well as the column heater (318 K) are located in the 820 IC separation center. For conductivity detection a 833 liquid handling unit (suppression of the eluent) and a 819 IC detector are connected to the outlet of the column. For UV/VIS detection the outlet is connected to a 844 UV/VIS compact IC of which only the detector cell is used. To perform anion separation, aqueous sodium acetate and sodium carbonate eluents are used at a concentration range of 10–40 mM and a pH in the range of 3.00–8.40.

### 3. Results and discussion

#### 3.1. Preparation of zwitterionic exchangers

The preparations of all zwitterionic sulfobetaine precursors with one to five methylene groups between the charged functionalities are done by simple nucleophilic substitutions. The five precursors can be synthesised in two to three stages starting from readily available starting materials [24]. The precursors carry a quaternary amine and a sulfonic acid functionality at certain distances between the charges. The rest of the different precursors – including the spacer – is identical. In order to enable attachment to the core material the zwitterionic moiety is attached to a hydrophobic styrene derivative as a spacer. The bonding reaction is a grafting reaction, by which selected amounts of zwitterionic precursors – and by this selected amounts of functional groups – can be attached to the core material. This is schematically shown in Fig. 2.

The utilised grafting reaction [25] allows the variation of the exchange capacity by varying the amount of zwitterionic precursor used in this reaction. This allows the synthesis of five zwitterionic exchanger materials of identical capacities with only one difference – the chain length between the charged groups.

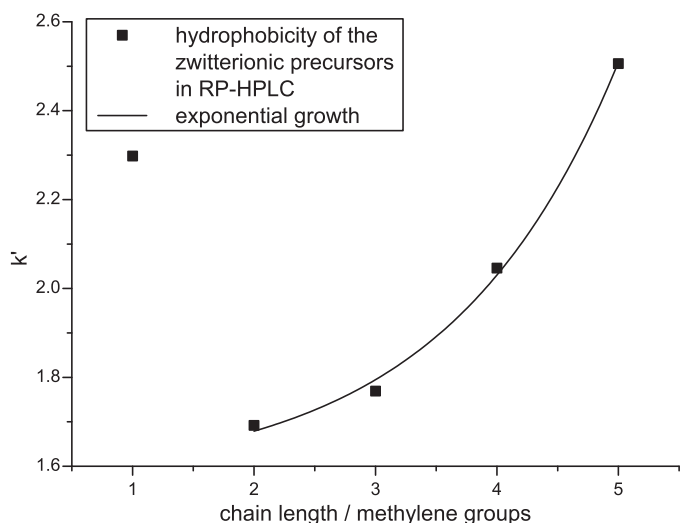
The spacers to the polymeric backbone, the core material, capacity as well as the nature of the covalent bonding are exactly the same for all prepared materials. Therefore the differences seen in the behaviour towards the separation of inorganic anions should only derive from the different distances between the charges.

#### 3.2. Determination of the zwitterionic precursor's polarities by RP-HPLC

To be able to get a closer look to the effective polarities of the prepared zwitterionic precursors (Fig. 1), the molecules are separated by RP-HPLC. The separations are performed using an aqueous eluent with 30% of methanol and a phosphate buffer at pH 3.0 under isocratic conditions. At low concentrations of the precursors, polarity should be strongly dependent on the ability of the precursors to compensate their charges via intramolecular interactions. Retention factors for the five prepared monomers are plotted versus the chain length in Fig. 3.

An exponential increase in retention factors can be found for the zwitterionic precursors with two to five methylene groups between the charged groups. Increasing retention means a decrease in hydrophobicity of the molecule. The precursor with two methylene groups between the ammonium functionality and the sulfonic acid is the most polar molecule out of the five investigated. This C2 distance seems to be long enough to separate the charges effectively. In addition, the distance of two methylene groups is short and inflexible enough to avoid intramolecular interactions. With increasing chain length, the molecules get more flexible with an increasing ability to compensate charges intramolecularly. The outcome is a reduction of the molecule polarity/hydrophobicity.

The only exception to this exponential increase is sulfobetaine C1, which exhibits a polarity between sulfobetaine C4 and sulfobetaine C5. In contrast to the long chained molecules, the precursor with one methylene group has a rather inflexible geometry with



**Fig. 3.** Retention factors of the zwitterionic monomers used as precursors. Column: Nucleosil C18 (250 mm × 4 mm); eluent: methanol/water/phosphate buffer (pH 3.0) 30:50:20; flow rate: 1.0 ml/min; detection: UV at 210 nm.

only a one methylene group distance between the charged groups. This distance seems to be that short, that the charges get compensated intramolecularly, which leads to a lower total polarity of the molecule.

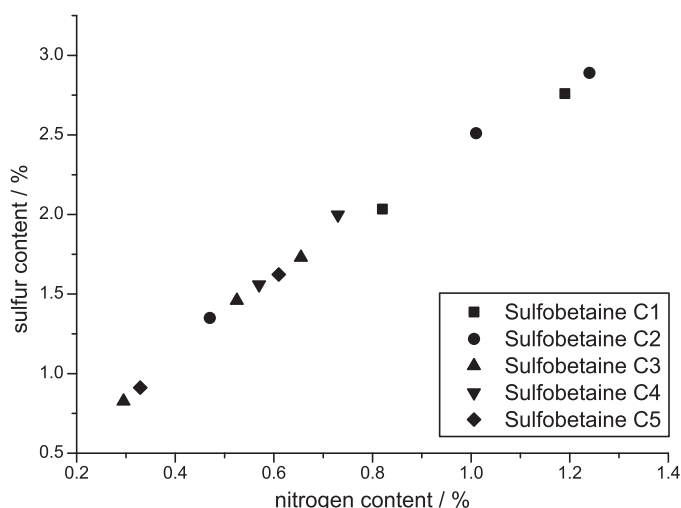
### 3.3. Determination of exchanger capacities

Beside the polarity the control and adjustment of capacities is of major interest for the investigation of chain length effects on zwitterionic exchangers. The utilised grafting reaction allows an adjustment of the capacity, but also a reproducible method for the determination of the capacities of the prepared materials is still required. In cation and anion exchange, it is common to determine capacities dynamically.

Unfortunately, dynamical methods cannot be applied to zwitterionic materials. Zwitterionic materials show intra- and intermolecular saturation of their charges, so attached anions as well as cations will be flushed off the column using water as the eluent which is applied as a necessary purging step in dynamical methods. Therefore a different method for determination of capacity must be found. Whereas dynamical methods provide “effective capacities” which means they show only the amount of functional groups available for analytes, non-dynamical methods will represent the total or bulk capacity of the whole resin.

One method to get this bulk capacity is elemental analysis. Elemental analysis was carried out for all prepared exchangers determining the amount of sulfur as well as the amount of nitrogen. The special advantage of this method is that nitrogen capacity (quaternary amines) and sulfur capacity (sulfonic acids) can be compared and should be identical. Both sulfur and nitrogen contents determined by elemental analysis are compared in Fig. 4. In this figure, the sulfur and nitrogen contents of stationary phases with different capacities are plotted for each of the five prepared sulfobetaines.

Both elements show a good correlation with a linear relationship over a wide range of sulfur and nitrogen contents. The mass percentages of sulfur are a little more than twice as high as the mass percentages of nitrogen. This means that both elements are present in the same molar quantity. By this it is verified, that zwitterionic groups were attached by the utilised grafting reaction. The disadvantage of elemental analysis is its duration. Therefore a second method for the determination of sulfur based on X-ray fluorescence was developed.



**Fig. 4.** Correlation between the sulfur and nitrogen contents of the synthesised exchangers. Contents determined via combustion elemental analysis.

The matrix matched calibration standards for the XRF method were prepared by suspending unfunctionalised polymeric resin in acetone/ammonium sulfate of known concentration and removal of the liquid at 338 K. Both methods give comparable results for the capacities of the sulfobetaine materials from one to five methylene groups showing a good correlation with a linear relationship over a wide range of capacities. A variation of the amount of zwitterionic precursor used for the grafting reaction allowed the preparation of five materials with almost identical capacities (Table 1). The difference in capacities is about six percent regarding highest and lowest capacity and therefore negligible.

In addition to this, the capacities of the ZIC-HILIC and ZIC-pHILIC materials were determined using the same techniques. They were found to be 186  $\mu\text{eq g}^{-1}$  (ZIC-HILIC) and 201  $\mu\text{eq g}^{-1}$  (ZIC-pHILIC), which means they are 43% respectively 54% higher than the capacities of the prepared series of stationary phases. Although not having comparable capacities and different core materials, these zwitterionic exchangers were analysed as well known benchmarks, especially since they carry the same functional group as sulfobetaine C3.

### 3.4. Separation of inorganic anions using acetate eluents

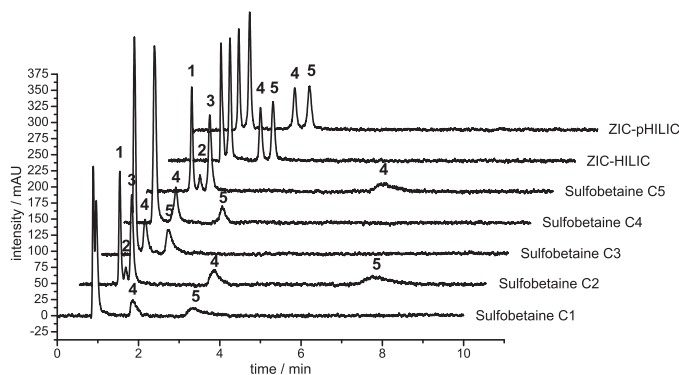
Separations of five inorganic anions using sodium acetate eluents are carried out for the characterisation in the ZIC separation mode. Sodium salts of bromide, iodide, thiocyanate, nitrite and nitrate, all of them detectable by UV at 210 nm, are used as analytes. Retention of these analytes can be achieved with all five prepared materials as well as with the commercial exchangers. The chromatograms are shown in Fig. 5.

It can be seen that baseline separation of iodide and thiocyanate under the given conditions is achieved by all seven materials, whereas the separation of nitrite, bromide and nitrate can only be observed for two (sulfobetaine C2) respectively five (sulfobetaine C5) methylene groups between the charges. In addition to this, it

**Table 1**  
Capacities of the five prepared materials with varying distance between the charged groups.

Chain length/methylene groups	1	2	3	4	5
Capacity <sup>a</sup> ( $\mu\text{eq g}^{-1}$ )	133	130	133	126	125

<sup>a</sup> Capacities were found by determination of sulfur contents using X-ray fluorescence.



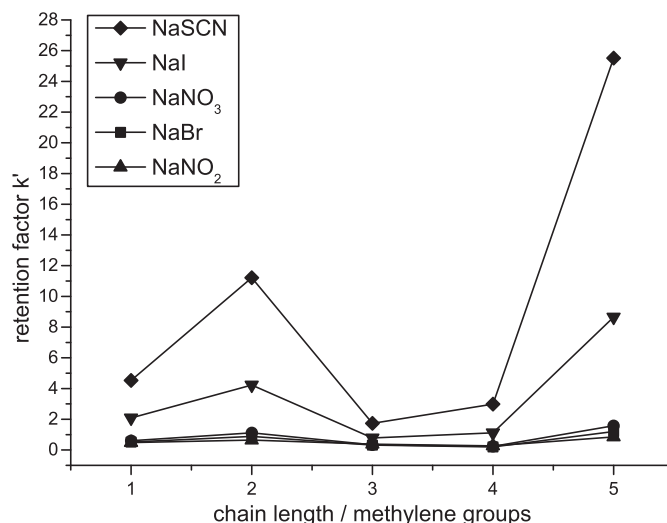
**Fig. 5.** Separation of inorganic anions. Analytes: nitrite (1), bromide (2), nitrate (3), iodide (4) and thiocyanate (5). Eluent: 40 mM sodium acetate, pH 4.75; flow rate: 1.0 ml/min, UV detection at 210 nm, 318 K.

is obvious, that the ability to retain inorganic anions is affected by the chain length of the functional group. While the prepared material with three methylene groups (sulfobetaine C3) show the lowest retention times for all analytes, with nitrite, bromide and nitrate coeluting, sulfobetaine C5 exhibits that much retention, that thiocyanate does not elute within 10 min. The commercially available columns ZIC-HILIC and ZIC-pHILIC allowed the baseline separation of iodide and thiocyanate, but both show coelution of nitrite and bromide in the first peak. The difference in retention factors between sulfobetaine C3 and the ZIC-HILIC and ZIC-pHILIC materials is evident and due to the different capacities, whereas the ZIC-HILIC (silica core) and ZIC-pHILIC (methacrylate core) give almost identical chromatograms. Under these conditions the influence of the core material seems to be negligible.

The dependency of anion retention on interchange chain length can be visualised by plotting retention factors versus chain lengths as shown in Fig. 6. Looking at this plot one should keep in mind that the series of prepared materials have the same capacities, identical spacers to the polymeric backbone as well as identical core materials. Under the selected conditions (40 mM sodium acetate, pH 4.75) sulfobetaines C3 and C4 as well as the commercially available materials with three methylene groups show the lowest anion retentions of all tested materials. Although having lower capacities, the sulfobetaines C2 and C5 show higher retention factors than the ZIC-HILIC and ZIC-pHILIC phases.

### 3.4.1. Influence of the eluent ionic strength on the separation of inorganic anions

After having shown the chromatograms for the anion separations, eluent composition is varied. First step of these variations is the ionic strength of the sodium acetate eluent while holding the pH constant. The shown difference in retention factors (Fig. 6) also becomes obvious when  $k'$  is plotted versus the sodium acetate concentration as shown in Fig. 7. All separations are carried out at a pH of 4.75. The commercial exchanger materials as well as the sulfobetaines C1 and C5 show the expected behaviour. According to the proposed mechanism in ZIC [22], which was determined using dynamically coated C18-phases, the formation of an electrical double layer (EDL) leads to increasing retention factors with increasing ionic strength of the eluent resulting in an asymptotical approximation to a saturation limit. Once the EDL is formed completely, a further increase in ionic strength does not have any effect on retention factors. Although not being dynamically coated materials sulfobetaines C1 and C5 as well as ZIC-HILIC and ZIC-pHILIC stationary phases show the same behaviour. For the sulfobetaines C1 and C5 the  $k'$  values plotted in Fig. 6 are close to the maximum retention factors. This difference compared to the commercial materials was expected looking at the lower capacities of the prepared sul-



**Fig. 6.** Dependence of the anion retention factors on the distance between the charged groups.

fobetaine exchangers. With lower capacities the saturation of the EDL should be reached at lower eluent ionic strength, therefore the maximum retention factors are reached earlier.

In contrast to this, the materials with two, three and four methylene groups between the charges behave different. Retention factors on the sulfobetaine C2 remain almost constant at quite high absolute values. The reason can be found when looking back on the previously determined hydrophobicities of the zwitterionic precursors (Fig. 3). This experiment showed, that sulfobetaine C2 was the most polar compound of the homologous row. This effect was previously explained by the C2 distance between the charges, which is too short and inflexible to allow intramolecular saturation of the charges. This explanation can also be used to explain its behaviour regarding influence of ionic strength. Due to the inflexibility of the functional groups, the geometrical alignment of these groups is rather fixed. Intramolecular interactions, that are found for the sulfobetaines C1 and C5 and that are reduced with increasing ionic strength, do not take place for sulfobetaine C2. Therefore no effect can be seen with increasing the eluent ionic strength. At the same time it can be stated, that separation of the anions on this stationary phase also is not a classical ion exchange mechanism. Otherwise retention factors would decrease with increasing eluent ionic strength.

So the mechanism is still a ZIC mechanism but due to the forced linear alignment of the functional groups caused by the C2 distance, the accessibility of the anion exchange site is always given. Therefore retention factors are independent from eluent ionic strength in the range of ionic strength tested. This leads to the assumption, that accessibility of the ammonia functionality by linearisation of the zwitterionic groups might be a better explanation of the ZIC mechanism. The formation of an EDL leads to a linearisation of the functional groups of zwitterionic exchangers with longer interchange chain lengths. Therefore the EDL theory was a proper way to explain the observations made using dynamically coated C18-materials with three methylene groups between the charged centers. But from sulfobetaine C2 we can see, that the linear alignment of the functional groups is the real cause for the observed behaviour.

In contrast to sulfobetaine C2 the curves for sulfobetaines C3 and C4 show an almost linear increase in retention factors with increasing eluent ionic strength. No asymptotic approximation to a retention factor limit can be seen for the investigated ionic strength as it was expected and found for the sulfobetaines C1, C5 and the

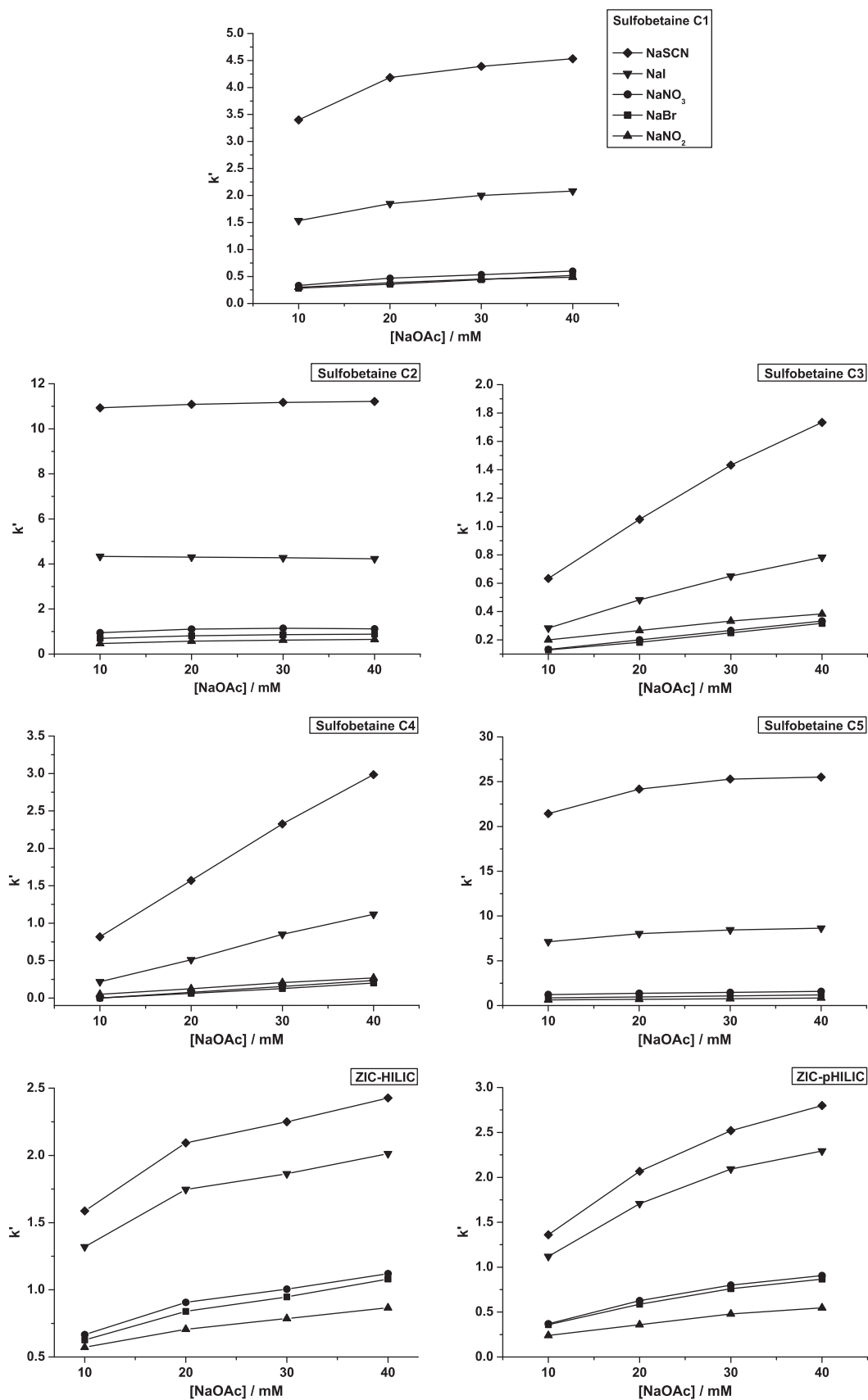


Fig. 7. Influence on retention by eluent ionic strength. Eluent: sodium acetate, pH 4.75; flow rate: 1.0 ml/min, UV detection at 210 nm, 318 K.

ZIC-HILIC and ZIC-pHILIC exchangers. Looking closely at the data points at an eluent ionic strength of 40 mM (C3 and C4 in Fig. 7) a beginning decrease in slope can be assumed. Therefore sulfobetaines C3 and C4 seem to behave as it was expected for ZIC materials, but the amount of eluent ions needed to reach saturation of the EDL or linearisation of the functional groups is much higher than for the other materials. Due to the limited UV-transparency of even higher concentrated sodium acetate eluents, this assumption cannot be confirmed experimentally.

At least it has to be stated, that – although having the same functional groups as the ZIC-HILIC and ZIC-pHILIC phases – the C3 exchanger behaves different. As stated before, the influence of the core material is negligible. Therefore, to explain this different behaviour, the capacities of the examined materials have to be considered. Regarding the lower determined bulk capacities a linearisation of the functional groups should appear at lower concentrations for the sulfobetaines C3 and C4 compared to the ZIC-HILIC and ZIC-pHILIC exchangers. An asymptotical approximation to the saturation limit should appear at lower concentrations as well, but experimental data is inverted. So the bulk capacities are not crucial for the observed differences.

The unexpected behaviour of the sulfobetaines C3 and C4 might then be explained by looking at the distances between adjacent functional groups on the surface of the core materials. Not knowing the density of occupation of the core material surface with functional groups, this generally can only be an assumption, but determining this density of occupation is almost impossible regarding covalently bond functional groups. Using dynamically coated C18-phases, the areas occupied by zwitterionic tensides and therefore the distance between the functional groups could be calculated from adsorption isotherms of these tensides. Nesterenko et al. determined the distances between zwitterionic tensides on a completely coated surface with a homogeneous mono-layer distribution of the adsorbed molecules to be in the range of 10.4–11.6 Å depending on the tenside used [23]. This distance is in the range of the interchange distances. Obviously dynamically coated materials with C18-cores cannot be compared directly to covalently bond stationary phases. But Nesterenko et al. at least showed that functional groups can occupy surfaces in a quite dense way. Therefore it is possible, that covalently bond functional groups can have a comparable density of occupation.

Assuming a high density of functional groups concerning the examined commercial exchangers, differences between sulfobetaines C3/C4 and these commercial materials can be explained. The ZIC-HILIC and ZIC-pHILIC exchangers have slightly higher capacities than the PS/DVB materials. By this the functional groups of the commercial phases might be close enough together to provide intermolecular interactions between adjacent functional groups. Having lower capacities, the functional groups of sulfobetaines C3 and C4 do not provide these interactions and therefore have an increased tendency to form inner ion pairs. Especially for these two sulfobetaines the ion pairs formed would be six and seven ring species, which are expected to be very stable. Due to the intermolecular interactions taking place on the commercial materials, intramolecular interactions are hindered, linearisation of the functional groups can be achieved easily. The stability of the inner ion pairs of C3 and C4 make them hard to linearise and therefore a higher eluent ionic strength is needed to reach linearisation of the functional groups. By this – although having lower capacities and leading to lower retention factors – an approximation to the retention factor limit is reached at higher ionic strength for the sulfobetaines C3 and C4. Apart from that these two exchangers show the typical ZIC behaviour.

Besides this comparison of the sulfobetaines C3/C4 with the commercial exchangers, the differences between the series of sulfobetain exchangers are interesting. Although having the same

capacities, the five materials do not show a linear behaviour looking at Fig. 6, even at much higher ionic strengths of the eluent.

In addition to that the comparison between sulfobetaine C1 and sulfobetaine C5 leads hopefully to a better understanding of the mechanisms in zwitterionic ion chromatography. Cook et al. [11] proposed the formation of a Donnan membrane having an influence on ion separation in ZIC using dynamically coated stationary phases. For sulfobetaine C1 with a rigid alignment of the short chained functional groups, a similar Donnan membrane might be localised as rigid as the functional groups themselves. Looking at the sulfobetaine C5 this should not be the same. Sulfobetaine C5 has very flexible functional groups with large interchange distances. The formation of a Donnan membrane for this alignment should not be that fixed, maybe the proposed membrane even does not form for this distance. The lack of such a membrane or at least the lower potential of the membrane would explain the extremely high retention factors observed for this exchanger concerning anion separation. A Donnan membrane being less strong will not hinder anions as effectively as it can be seen for shorter distances. This also might be an additional explanation for the behaviour of sulfobetaines C3 and C4.

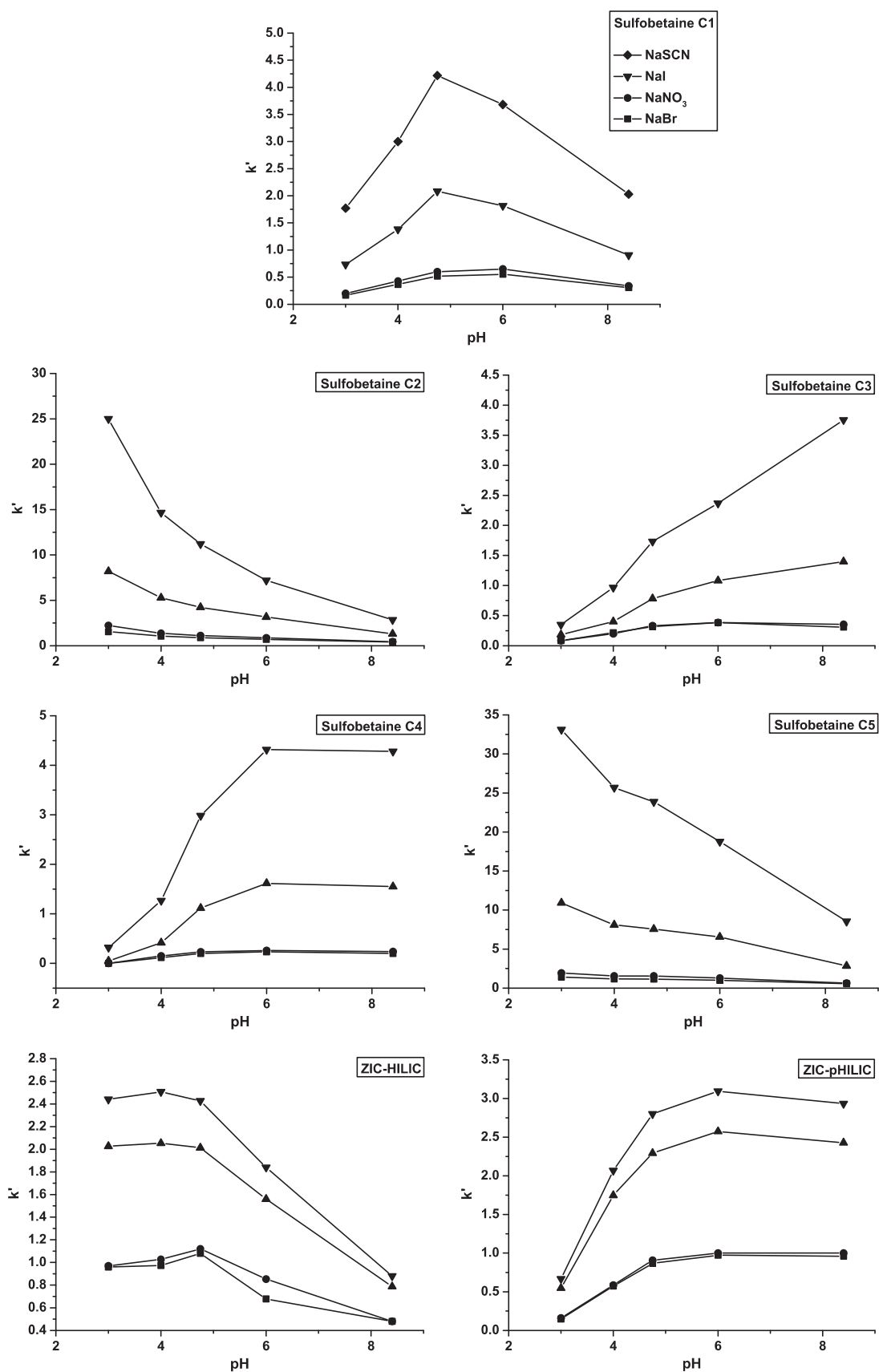
#### 3.4.2. Separation of inorganic anions with varying eluent pH

Another factor expected to have an influence on the retention factors is the eluent pH. Therefore the retention of inorganic anions using 40 mM sodium acetate eluents with pH in the range of 3.00–6.00 was determined. The ionic strength of the eluents utilised corresponds to the highest concentration in Fig. 7. The highest pH of 8.4 is realised using a 40 mM sodium hydrogen carbonate eluent. The results are shown in Fig. 8. Nitrite is not shown in this figure due to the  $pK_a$  of nitrous acid (3.4) which has an influence on retention factors at pH in the range of 4.00–3.00.

Again the results for some of the prepared exchangers are similar, while others show appreciable differences. The sulfobetaine C1 shows a unique behaviour, while plots for sulfobetaine C2 and C5 are quite similar. Similarities can also be found for the sulfobetaines C3 and C4. The commercially available materials ZIC-HILIC and ZIC-pHILIC show comparable behaviours as well, while they give completely different results compared to the sulfobetaines C2/C5 and C3. The curves of the ZIC-pHILIC material have analogies to the curves of the C1 and C4 sulfobetaines.

The C1 exchanger – as mentioned before – has a rigid coordination of the exchanger groups, which should cause the different behaviour of this material. At a low pH retention factors are low and growing with increasing pH. The maximum is reached near pH 4.75 and decreases in both directions to almost equal retention factors at pH 3.0 and pH 8.4 (hydrogen carbonate eluent).

A change in eluent pH from 3.00 to 6.00 renders the hydron/hydroxide ratio as well as the amount of acetate compared to the amount of acetic acid. The hydron concentration is lowered whereas the hydroxide concentration is increased. In addition to that, the sodium acetate concentration is increased. An increase in acetate concentration should result in curves as the ones shown in Fig. 7. A 40 mM sodium acetate eluent at pH 3.00 has fewer acetate ions in it than a 10 mM sodium acetate eluent at pH 4.75. Hence, the increase in retention factors from pH 3.00 to 4.75 can be explained. With further increase in eluent pH a reduction in retention times can be observed. From pH 4.75 to 6.00 the sodium acetate concentration increases, but retention factors are reduced. If the behaviour of the C1 exchanger was stringent, a further increase in retention factors would be expected, but the change in sodium acetate concentration does not seem to have an influence at higher pH. Therefore the reason for the decrease in retention factors can only be seen in the increase in hydroxide concentration or the decrease in hydron concentration.



**Fig. 8.** Influence on retention by eluent pH. Eluents: sodium acetate and sodium carbonate (pH 8.4). Ionic strength: 40 mM; flow rate: 1.0 ml/min, UV detection at 210 nm, 318 K.



Cook et al. [11] investigated dynamically coated materials and gave an explanation for changing retention factors with changing concentrations of different eluent ionic species. In their work, they examined cation retention on zwitterionic exchangers with an outer positive charge and an inner negative charge, which is opposite to the exchangers examined here. They argued the formation of positive or negative Donnan membranes, depending on how strong the shielding of the positive and negative functional group is. Different eluent anions and cations have different shielding effects according to the Hofmeister chaotropic series [26]. For our exchangers, the same argument can be used regarding the location of the charges. With identical cations, namely sodium, the Donnan membrane should become least negative or even positive, if the mobile phase anion only weakly shields the inner positive group. By increasing the concentration of hydroxide, which is even more weakly shielding compared to the competing acetate, the shielding of the inner positive group should be reduced which should result in a weak negative or more positive Donnan membrane. Retention factors for the analyte anions should increase, because they should more easily be able to reach the inner ammonia group.

The effect we observed is inverted, with a decrease in retention factors with increasing pH which cannot be explained by the increase of hydroxide concentration. However we still have to keep in mind, that a change to higher pH not only means an increase in hydroxide concentration but also a decrease in hydron concentration. Cook et al. did not examine  $H^+$ , because they say it is a special case. Hydron was investigated by Hu et al. [27] who found, that  $H^+$  was highly retained on dynamically coated stationary phases with inner cation exchange groups, which means that the hydron is shielding the negative charge strongly. A better shielding of the negatively charged sulfonic acid of the sulfobetaine C1 by  $H^+$  will lead to a less negative or more positive charged Donnan membrane, which in the end leads to a stronger retention of anionic analytes at low pH. At a pH in the range of 4.75–8.40, the  $H^+$  concentration decreases which results in a decrease in retention factors due to the formation of a less positive or more negative charged Donnan membrane which explains the observed behaviour. At a pH in the range of 3.00–4.75 the effect of increasing acetate concentration outweighs the influence of the Donnan membrane. The reason for this is, that the oppositely charged functional groups of the sulfobetaine C1 compensate each other at low eluent ionic strength due to the short interchange chain length. With directly compensated charges, the formation of Donnan membranes is hindered, while the direct compensation is reduced with increasing eluent ionic strength.

The sulfobetaines C2 and C5 show decreasing retention factors with increasing pH, which is similar to the behaviour of sulfobetaine C1 at pH values higher than 4.75. As shown before, using the C2 material, the retention factors are independent from eluent ionic strength. For this stationary phase a decrease in retention times can only be caused by hydron/hydroxide concentrations. A decreasing hydron concentration resulting in a decreasing shielding of the sulfonic acid functionality leads to decreasing retention factors over the whole range of pH. By this behaviour of the sulfobetaine C2 it can be proven, that Donnan membranes according to Cook et al. [11] also exist on covalently bonded stationary phases. The influence of the shielding of exchange functionalities by eluent ions is similar to the effects observed using dynamically coated materials.

Contrary to the sulfobetaine C2, the sulfobetaine C5 shows a  $k'$  versus  $[NaOAc]$  plot that is quite similar to the plot for the C1 material (Fig. 7). Retention factors of this phase are dependent on eluent ionic strength. Anyway the effect of eluent pH on sulfobetaine C5 is more similar to C2. A decreasing  $H^+$  concentration reduces retention factors. The effect of Donnan membranes outweighs the linearisation of the functional groups by eluent ionic strength for this stationary phase. The reason for this must be found

in the long interchange chain length. As stated before, the prepared series of stationary phases does not provide the ability of intermolecular interactions between adjacent sulfobetaine groups. In addition to this, the sulfobetaine C5 does not form stable inner ion pairs which results in a retention factor limit that is reached at quite low eluent ionic strength (Fig. 7). The sodium acetate eluent used for the variation of eluent pH has a high eluent ionic strength by what linearisation of the functional groups is already reached for the sulfobetaine C5. Therefore the shielding of the sulfonic acid by  $H^+$  is the main effect that can be seen.

Completely different results are found for the sulfobetaines C3 and C4. As mentioned before, both materials have not yet reached saturation concerning the EDL or the linearisation of the functional groups at 40 mM of sodium acetate at a pH of 4.75. This was due to the formation of stable inner ion pairs and the lack of the ability to saturate charges intermolecularly. With changing eluent pH, these materials behave as they did with changing eluent ionic strength. The effect of linearisation of the functional groups outweighs the formation of Donnan membranes. Obviously Donnan membranes can only form on stationary phases that show at least a minimum of symmetry. Due to the formation of inner ion pairs the functional groups are bent and do not show a linear alignment. This hinders the formation of Donnan membranes, the shielding effect by  $H^+$  is not important in the range of pH tested.

In the end concerning the commercially available columns, no stringent results can be observed. Both materials show plots that seem to be more or less a mixture of previously shown effects. Their behaviour can be compared to the behaviour of sulfobetaine C1, having maxima of  $k'$ , although the commercial materials have the same functional groups as sulfobetaine C3. Contrary to sulfobetaine C3 for the ZIC-HILIC and ZIC-pHILIC materials linearisation of the functional groups does not completely outweigh formation of Donnan membranes. The reason for this is the earlier stated higher density of functional groups on the exchanger's surface due to the higher bulk capacity of the commercial materials. This higher density provides the ability of saturating charges of adjacent functional groups by intermolecular interactions. Therefore the formation of inner ion pairs is reduced. As shown in Section 3.4.1, by this the complete linearisation of the functional groups is reached at lower eluent ionic strength.

This is what happens at a pH in the range of 4.50–5.00 for both commercial exchangers. In this range of pH the linearisation of the functional groups is reached. Therefore, the stationary phases reach the required symmetry to provide the formation of Donnan membranes. At a pH in the range of 4.75–8.40 the reduced shielding effect by the decrease of  $H^+$  concentration occurs. As a result the graphs for variation in eluent pH show competitive effects of the influence of eluent ionic strength and the formation of Donnan membranes depending on the degree of linearisation of the functional groups.

Due to the different core material, this specially affects the ZIC-HILIC phase. For the ZIC-HILIC exchanger not only the shielding of the sulfonic acid is reduced at higher pH, but also the dissociation of residual silanols in this silica based stationary phase occurs. This changes the polarity and the whole electronic structure of the core material which results in the distinct reduction in retention factors.

#### 4. Conclusion

A series of sulfobetaine exchangers with identical core materials, identical spacers to the polymeric backbone as well as comparable capacities allows the examination of the influence of interchange chain length on the separation of inorganic anions. It was found, that the zwitterionic precursor with two methylene groups between the charges is the most polar compound of all.

This leads to a unique behaviour concerning the influence of ionic strength on anion retention, using the material grafted with this C2 precursor. Sulfobetaine C2 shows constant retention factors with changing sodium acetate concentrations, over a concentration range of 10–40 mM. This leads to a new interpretation of the ZIC mechanism by stating that linear alignment of the functional groups is more important than the formation of an EDL. It could also be found, that the sulfobetaines C3 and C4 reach the retention factor limit at higher eluent ionic strength although having lower capacities than the commercially available ZIC-HILIC and ZIC-PHILIC exchangers from Merck SeQuant. This was expected to be due to a lower density of functional groups on the surface of the prepared series of sulfobetain exchangers resulting in distances between adjacent functional groups that are too big to allow intermolecular interactions. In addition the formation of a Donnan membrane could be investigated and confirmed clearly by the results observed using the sulfobetain C2. A special influence of H<sup>+</sup> on the exchanger's ability to separate inorganic anions due to the shielding effect of hydron on the sulfonic acid functionality could be found. Especially the sulfobetaines C2 and C5 are found to be highly capable of separating inorganic anions while also showing a strong influence of H<sup>+</sup> concentration on retention factors.

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