Separation of α-Amino Acids Using a Series of Zwitterionic Sulfobetaine Exchangers

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Abstract

A set of five new covalently bond sulfobetaine exchangers with inner quaternary amines and outer sulfonic acids have been prepared by attachment of a series of zwitterionic precursors to hyperporous divinylbenzene polymers using a grafting reaction. The series of zwitterionic exchangers have the same backbone and identical spacers to the polymeric backbone, as well as comparable capacities. The only difference is the chain length for one to five methylene groups between the charged functional groups. Chromatographic properties are examined by separation of α-amino acids using sodium acetate and nitric acid eluents. The separation mechanism is explored by varying eluent ionic strength and eluent pH, resulting in the conclusion that amino acids are separated due to cation exchange interactions. This is a behavior never before observed using zwitterionic exchangers. It contradicts the fact that sulfobetaine-type materials used in zwitterionic ion chromatography (ZIC) usually are well suited for anion separation and only poorly for cation separation. Contrary to anion separations using the identical set of exchangers, the materials with three and four methylene groups between the charges give the highest retention factors. Materials showing the high potential in ZIC separations of inorganic anions give low retention factors for amino acids and vice versa.

Introduction

The separation and detection of amino acids has been a challenging task for a long period of time. Cation exchange chromatography, reversed phase liquid chromatography (RP-LC) coupled to UV absorbance, or fluorescence detection have been the standard methods for separating and detecting amino acids for decades. Unfortunately, these methods require pre-column or post-column derivatisation of the analytes (1). Therefore, large numbers of different analytical methods have been developed in recent years and still new techniques have been published to complement current methods and achieve improvements in separation and detection.

One of the most widely applied techniques for amino acid separation is cation exchange chromatography using a lithium buffer system, followed by post-column derivatisation with ninhydrin and UV detection (2). Separation using cation exchange chromatography gives the advantage of not needing post-column derivatisation to achieve amino acid retention. New attempts allow the simultaneous determination of UV-detectable urinary creatinine and several amino acids by using eluents with a low UV-absorbance and a low-capacity cation exchange resin as the stationary phase (3). Contrary to cation exchange separations, techniques like RP-high-performance LC (4) or gas chromatography (GC) (5) can only be accomplished by precolumn derivatisation with the need for intense sample pretreatment. This again allows enantioseparation of amino acids using chiral GC columns or chiral derivatisation reagents (6,7).

Although these approaches lead to good separations and have special advantages, in general methods with reduced sample preparation would be preferable. Therefore, new separation mechanisms have been applied in recent years. One of the most promising and fast growing methods is hydrophilic interaction chromatography (HILIC). Amino acids are being used as analytes with most of the commonly examined HILIC phases (8). One of the advantages of HILIC separations is the mobile phase composition. Often sodium acetate eluents with acetonitrile contents of 60% to 90% are used. These conditions are perfectly suited for coupling with ESI-MS detection. This detection method greatly improves sensitivity for amino acids compared to UV detection while HILIC separations are carried out without the need of pre column derivatisation (9). Besides pure HILIC separations, mixed mode separations using both HILIC and cation exchange mechanisms are used (10).

In recent years, there have been studies using zwitterionic exchangers to achieve the separation of amino acids. In general, zwitterionic materials are widely used for zwitterionic ion chromatography (ZIC) in combination with HILIC (ZIC–HILIC). In this mode, zwitterionic exchangers are known to be well suited for separation of polar organic analytes (11). Only two recent publications, however, deal with the ZIC–HILIC separations of amino acids (12,13). In addition to this, some work can be found in which zwitterionic exchangers are used to separate amino acids under ZIC conditions using pure water as the eluent (14).

In this work, the separation of amino acids was achieved by using a series of zwitterionic exchangers in ZICmode with eluents containing eluent ions without organic modifiers. Under these conditions, amino acids were expected to be separated in their zwitterionic form by multipoint interactions with the zwitterionic stationary phases. Sodium acetate and nitric acid eluents were examined and showed interesting separation

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mechanisms. The intercharge distance of the functional groups was expected to have an enormous influence on these separations. The results for amino acids were compared to separations of inorganic anions using the same series of exchangers under similar conditions (15). The commercially available ZIC–HILIC and ZIC–pHILIC exchangers by Merck SeQuant (Darmstadt, Germany) were tested as well to serve as well-known benchmarks to demonstrate the quality of the prepared series of exchangers.

Experimental

Reagents

Acetic acid (100%) and nitric acid (65%) were purchased from Carl Roth (Karlsruhe, Germany). Sodium hydroxide (50%) was obtained from Fluka (Buchs, Switzerland). Sodium acetate (≥ 99%) was purchased from J. T. Baker (Deventeer, Netherlands). α–Amino acids were purchased from Serva Feinbiochemica (Heidelberg, Germany). Ultrapure water having a resistivity of 18.2 MΩ was prepared using a Milli-Q water purification system (Millipore, Bedford, MA). Eluents and stock solutions of amino acids were prepared by dissolving the acids directly in ultrapure water. Reagents for the synthesis of the zwitterionic precursors were used in the highest available purity (16). The core materials of the prepared zwitterionic materials consisted of highly cross-linked macroporous polystyrene/divinylbenzene copolymer. The cross-linking degree was 55%, and the particle size was 4.6 µm. ZIC–HILIC and ZIC–pHILIC columns were obtained from Merck SeQuant (Darmstadt, Germany, column dimension: 100 mm × 4.6 mm i.d., 5 µm particle size).

Synthesis of zwitterionic precursors

The preparation of covalently bond zwitterionic exchangers is based on the synthesis of the corresponding zwitterionic precursors (16). On one hand, they provide the zwitterionic groups with the difference in intercharge chain length; on the other hand, the functionality for the attachment to the polymeric resin by a grafting reaction.

Therefore, zwitterionic precursors were prepared as aromatic monomers by simple nucleophilic substitution reactions between a styrene derivative as a spacer and tertiary amines. These tertiary amines with one to five methylene groups between the ammonia group 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 Figure 1.

Functionalization of polymeric particles by grafting

Functionalization of the resin was performed by a grafting reaction following a preparation invented by Raskop et al. (17). Thereby, the zwitterionic precursors were covalently bond to the polymeric resin. The grafting reaction is schematically shown in Figure 2. The stationary phases were packed using PEEK columns (100 mm × 4 mm i.d.).

Determination of capacities

The capacities for a series of five sulfobetaine type exchangers were determined by x-ray fluorescence analysis (XRF) (ARL Optim'X, Thermo Fisher, Waltham, MA). For this, 250 mg of the dried polymer were weighted on a sampling tray. The content of the sulfur was detected directly, measuring the sulfur $K\alpha_{1,2}$ line at 5.3731 Å with a pentaerythrit (PET) crystal.

Chromatographic conditions

All chromatographic separations were carried out using a Modular IC System (Metrohm AG, Herisau, Switzerland). For pumping the eluent at a flow rate of 1.0 mL/min, a 709 IC Pump was used. Injection of the samples was achieved by a 812 Valve Unit (20-µL injection loop). The column heater (308 K) as well as the disposable gold electrode for pulsed amperometric detection (PAD) were located in the 817 BioScan. Due to the acidity of the used eluents, a post-column addition of sodium hydroxide (500 mM, 0.3 mL/min) was necessary and achieved by another 709 IC Pump via a T-connector. The utilized pulse sequence for PAD was a modified version of the standard pulse sequence for sugar analysis. The pulse steps are given in Table I.

To perform amino acid separations, aqueous sodium acetate and nitric acid eluents were used. The sodium acetate eluents were at concentrations in the range of 10 mM to 40 mM and a pH in the range of 3.00 to 6.00. The nitric acid eluents were at concentrations in the range 10 mM to 100 mM. Void volumes were determined by using the solvent peak that occurs when injecting pure water blanks.

Table I. Pulse Sequence for the PAD of Amino Acids

Potential (V)	Duration (s)	Function
0.13	0.3	Equilibration of potential
0.13	0.1	Determination
0.75	0.2	Oxidative cleaning
–0.15	0.4	Reduction of gold surface

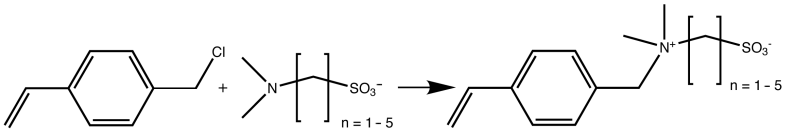

Figure 1. Synthesis of the zwitterionic precursors.

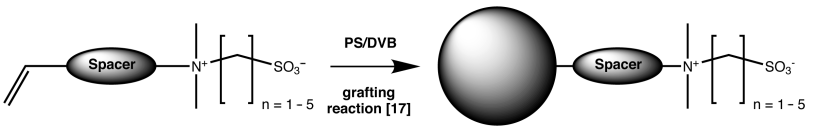

Figure 2. Scheme of the grafting reaction leading to zwitterionic exchangers.

Results and Discussion

Preparation of zwitterionic exchangers

The preparations of the zwitterionic sulfobetaine precursors with one to five methylene groups between the charged functionalities were done by simple nucleophilic substitutions. The five precursors were synthesized in two to three stages, starting from readily-available starting materials (16).

The five materials had quaternary amines with two methyl moieties, and a sulfonic acid moiety separated by different length methylene chains. The rest of the different molecules were identical. They were attached to a hydrophobic styrene spacer, which provided the attachment to the core material. The bonding reaction was a grafting reaction, by which the monomer precursors were attached to the core material. The grafting reaction is schematically shown in Figure 2.

The utilized grafting reaction (17) allowed for the variation of the exchange capacity by varying the amount of zwitterionic precursor used in the reaction. By doing so, the capacities were changed in a more or less linear way. This allowed for the synthesis of the five zwitterionic exchangers to be identical. The spacers to the polymeric backbone, the core material, the capacities, as well as the nature of the covalent bonding, were the same for all the prepared materials. This means that the differences seen in the behavior towards the separation of α-amino acids can only be derived from the different distances between the charges.

Determination of exchanger capacities

The capacities of the zwitterionic materials cannot be determined by dynamical methods as in anion or cation exchange chromatography. Therefore, non-dynamic methods giving the bulk capacity of the stationary phases had to be applied. In this work, capacities were determined by detecting the sulfur contents via XRF.

The matrix matched calibration standards for the XRF method were prepared by suspending unfunctionalized polymeric resin in acetone/ammonium sulfate of a known concentration and removal of the liquid at 338 K. A variation of the amount of the zwitterionic precursor used for the grafting reaction allowed for the preparation of five materials with almost identical capacities (Table II). The difference in capacities was approximately six percent regarding highest and lowest capacity and was therefore negligible.

The capacities of the commercial materials ZIC–HILIC and ZIC–pHILIC were determined as well. These columns had the same functional group as the prepared material, with three methylene groups between the charged sites, but differed in core material. The ZIC–HILIC exchanger was a silica-based phase, while ZIC–pHILIC was methacrylate-based. The capacities of these materials were 186 µeq/g (ZIC–HILIC) and 201 µeq/g (ZIC–pHILIC), thus being 43% and 54%, respectively, higher than the capacities of the prepared series of stationary phases. Although not having comparable capacities and different core materials, these zwitterionic exchangers were analyzed as well-known benchmarks to show the potential of the prepared materials.

Table II. Capacities of the Series of Zwitterionic Exchangers with Different Distances Between the Charged Groups

Chain length/methylene groups	1	2	3	4	5
Capacity/µeq/g	133	130	133	126	125

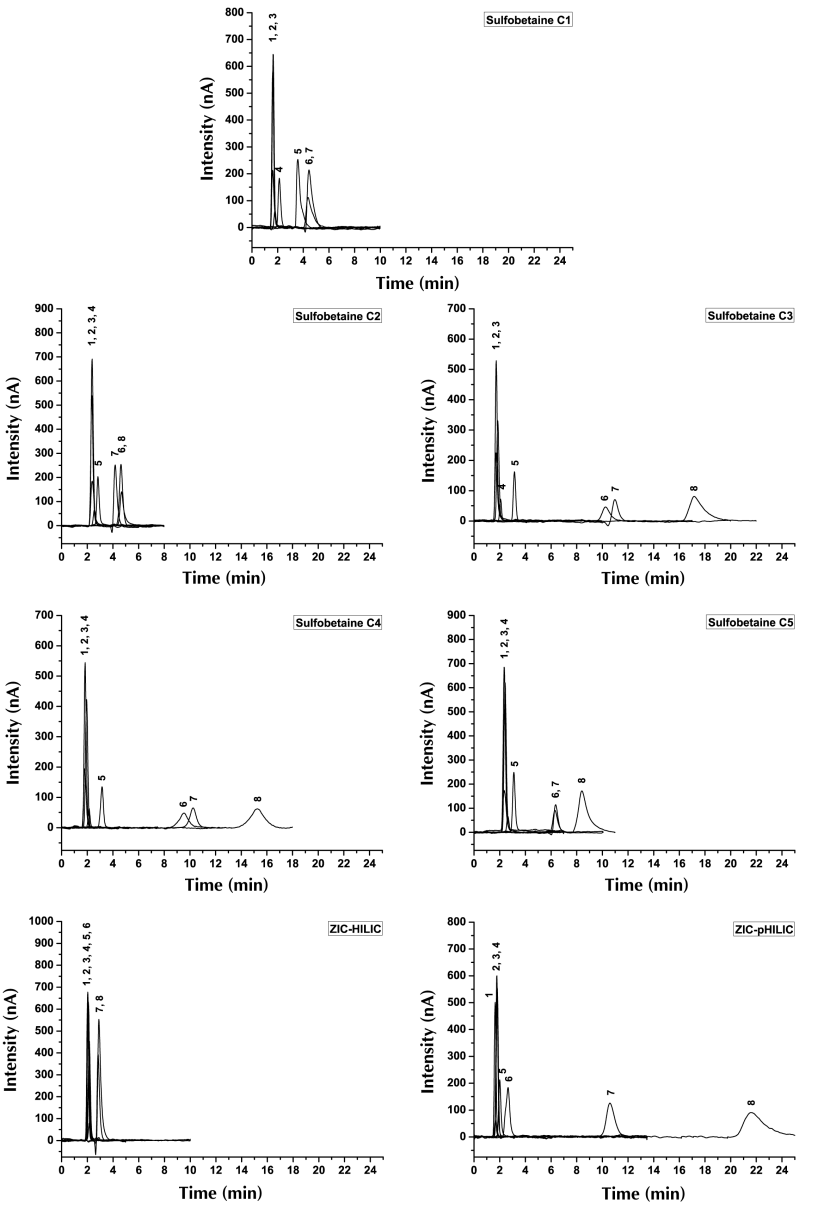

Figure 3. Chromatograms for the separations of eight α-amino acids using the series of sulfobetaine exchangers as well as the commercial columns. Analytes: 1, l-asparagine; 2, glycine; 3, dl-alanine; 4, l-proline; 5, dl-valine; 6, l-leucine; 7, l-lysine; 8, l-arginine. Eluent: 20 mM sodium acetate, pH 4.00, PAD; flow rate: 1.0 mL/min; temperature: 308 K.

Separation of α-amino acids using acetate eluents

Using this homologous row of sulfobetaine exchangers, α-amino acids could be separated by applying a sodium acetate eluent. The retention of eight amino acids was achieved with a sodium acetate eluent at a concentration of 20 mM with a pH of 4.00, while not all of the amino acids were separated. The chromatograms are shown in Figure 3. In addition to the chromatograms of the prepared exchangers, chromatograms of the commercially available materials ZIC–HILIC and ZIC–pHILIC are shown as well.

What can easily be seen are the remarkable differences between the chromatograms according to chain length between the charged groups. Such differences were expected to occur using amino acids as analytes. At a pH close to their pI's, the investigated α-amino acids were present as zwitterions having one methylene group between the carboxyl and the amino group. These zwitterionic analytes should be able to give multipoint interactions with the zwitterionic stationary phases. The exchanger with one methylene group between the ammonia group and the sulfonic acid (sulfobetaine C1) had a comparable distance between the charges and therefore was expected to give high retention times due to the formation of intramolecular multipoint interactions with α-amino acids.

In contrast to this assumption, the best separations can be achieved using the exchangers with three and four methylene groups between the charged groups (sulfobetaine C3 and sulfobetaine C4). Intramolecular multipoint interactions do not seem to play the expected important role. Furthermore, the choice of core material had an enormous influence on amino acid retention. This was best shown when comparing sulfobetaine C3 with the commercially available exchangers ZIC–HILIC (silica core) and ZIC–pHILIC (polymethacrylate core). Although the commercial materials had higher capacities in comparison to the prepared series of exchangers, they did not give remarkably higher retention times. The ZIC–HILIC column in particular only led to a group separation giving two peaks. The silica core reduced the functional group's ability to retain amino acids under the used conditions.

To get a better comparison between the synthesized materials with different chain lengths, the retention factors for all five exchangers are plotted versus the distance between the charged groups in Figure 4.

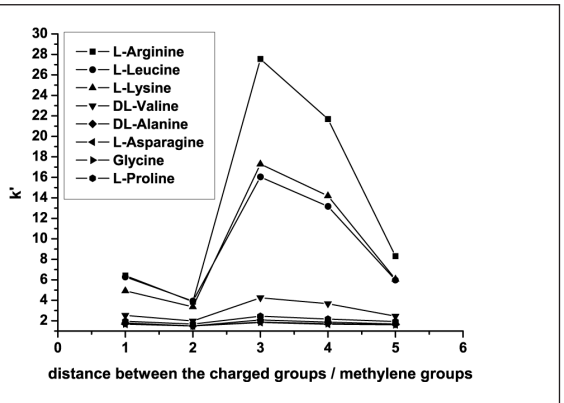

Figure 4. Dependency of amino acid separation on the distance between the charged groups.

The highest retention factors can be observed for sulfobetaine C3 and sulfobetaine C4. These results are contrary to the previously found correlation for the separation of inorganic anions under comparable conditions (15). In this work, sulfobetaine C2 and sulfobetaine C5 were found to give the highest retention factors. Therefore, observations concerning amino acid separation seem to be inverted compared to the results found for anion separations. The functional groups that provided good accessibility for anions did not provide good amino acid separations, and vice versa. To get a closer view into the mechanisms of the separation of amino acids, eluent conditions were changed systematically by starting with a variation of eluent ionic strength.

Variation of eluent ionic strength

ZIC-separations of inorganic anions with the prepared homologous row of exchangers showed the typical ZIC-behavior for most of the columns. In ZIC, using sulfobetaine type exchangers, anions were separated significantly better than cations (18). The retention factors for the anion separations increased with increasing eluent ionic strength, until they ended up in an asymptotical approximation to a saturation limit. This corresponds to the known mechanisms in ZIC (15,18). In comparison, the results for changing eluent ionic strength in amino acid separations are given in Figure 5 and appear quite different.

The eluent ionic strengths plotted are effective eluent ionic strengths. This means they are effective concentrations of eluent cations calculated for sodium acetate eluents with a certain pH (4.00 in all cases, determined by potentiometric pH measurement) and given total amounts of acetic acid plus sodium acetate using the Henderson-Hasselbalch equation. The values are already corrected concerning the dissociation of acetic acid at a given pH and a given total concentration.

Contrary to the observations made in anion separation under ZIC-conditions, separation of amino acids leads to a decrease in retention factors with increasing eluent ionic strength. This is a behavior well-known from ion exchange chromatography. Therefore the axes of the plots are formatted in a way that is common for ion exchange chromatography. The plots show two logarithmic axes giving almost linear relations for all of the exchangers except the ZIC–HILIC material. This commercially available exchanger shows very low retention factors in general. Therefore observations concerning this material should not be overestimated. The other materials only show slightly curved relations, which very much points to an ion exchange mechanism. This is a very interesting observation, because zwitterionic exchangers up to now are known to separate ions according to ZIC mechanisms. Due to the existence of a sulfonic acid as well as an ammonia group in one covalently bond moiety, the functional groups in general are able to compensate their charges via intra- or intermolecular interactions of the contrary charged groups. Therefore they usually inhibit classical ion exchange mechanisms.

In addition to this, the amino acids were expected to be present as zwitterions, thus interacting with the functional groups of the stationary phase as zwitterions by multipoint interactions. A reduction in retention factors with increasing eluent ionic strength is observed. To get a closer look into the behavior of amino acids, one should have in mind the general structure of

the analytes. All amino acids carry a carboxylic acid and a primary amine. Therefore, their charge can be negative, neutral (to the outside), or positive, depending on the pH. The pI's of all examined amino acids are between 5.41 and 11.76. These are the pH values at which the ionization degrees of both charged groups are equal. The examined pH is 4.00, hence slightly below the pI of most of the amino acids investigated. Under the chosen conditions, all of the examined amino acids must be present in a more cationic state. This leads to the assumption that the observed retention mechanism is likely to be a cation exchange retention mechanism.

This indeed is an unexpected observation for sulfobetaine exchangers. The mechanism not only seems to be an ion exchange mechanism, it also seems to be a cation exchange mechanism. As mentioned before, sulfobetaine type exchangers usually can hardly be used for cation separations. Amino acid separation seems to be a result of cation exchange. To validate these assumptions, further changes in eluent composition are carried out.

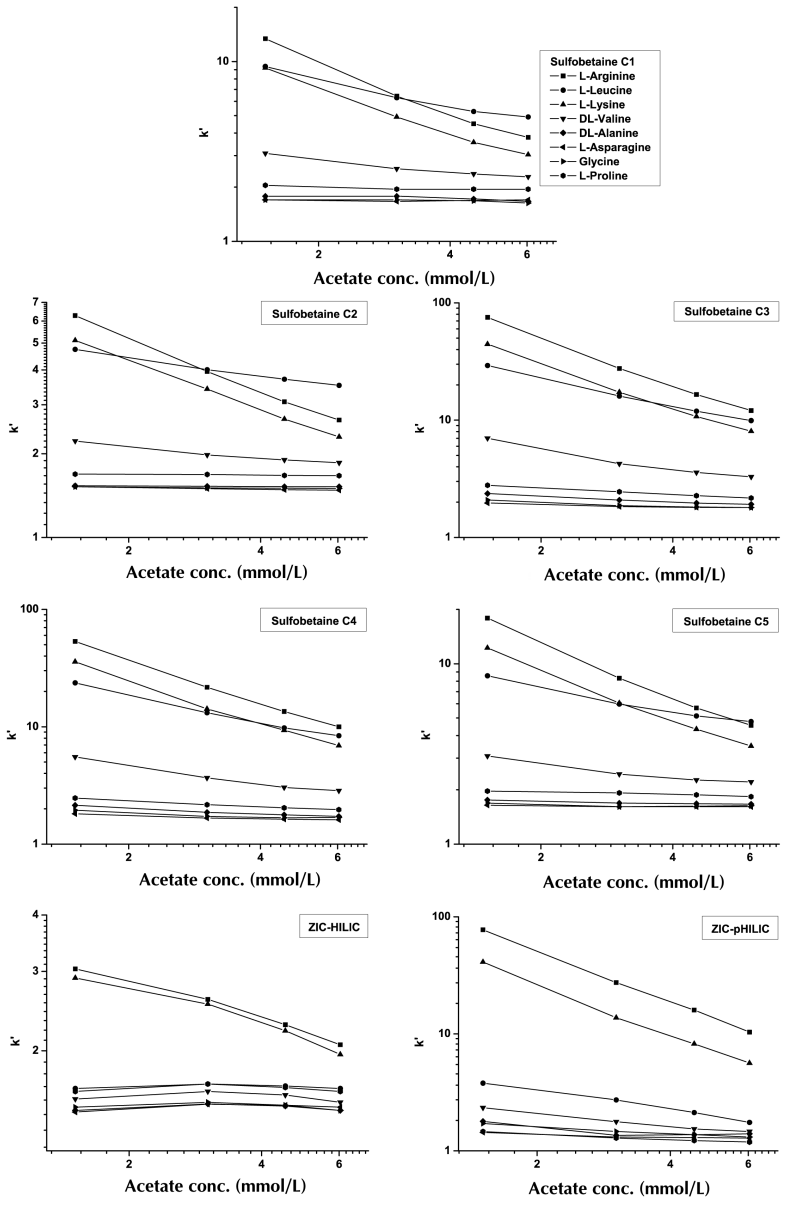

Figure 5. Plots of log k' vs. log ionic strength for all examined exchangers. Eluent: sodium acetate, pH 4.00. Concentrations are corrected by the dissociation of acetic acid in water using the Handerson-Hasselbalch equation.

Variation of eluent pH

The next change in eluent composition that can be applied is a change in eluent pH. This is shown exemplarily for sulfobetaine C3 in Figure 6. Results for the other sulfobetaine exchangers are comparable, with the exception of the ZIC–HILIC exchanger.

Figure 6 emphasizes the assumption that cation exchange is responsible for amino acid separation. Retention factors increased dramatically with the decreasing pH. The analytes with the lowest pI's (l-asparagine to l-proline) show the lowest increases in retention factors. With decreasing the pH, the amino acids were transformed into more cationic forms, and therefore experienced higher cation exchange interactions with the stationary phase.

This is contrary to preliminary assumptions concerning multipoint interactions, whether inter- or intramolecularly, and does not seem to be of any importance for these separations. If multipoint interactions were the key feature of amino acid separation on the investigated exchangers, the retention factors should be the highest for pH values close to the pI's of the analytes. Once the amino acids are present in their cationic form at low pH values they lack the ability of forming electrostatic interactions with both functional groups and therefore lack the ability of giving multipoint interactions. Due to the mesoporous PS–DVB core material with quite low capacities and therefore low surface densities of zwitterionic functional groups, the formation of intermolecular multipoint interactions might be hindered as well. Anyhow similar observations are made for the commercially available ZIC–pHILIC exchanger which has higher capacities than the prepared series of zwitterionic stationary phases.

The observed difference for the ZIC–HILIC exchanger, which does not change retention factors with changing pH at all, must be due to the used silica core.

Separation of α-amino acids using nitric acid eluents

Observations made for the prepared series of exchangers point out the assumption that cation exchange is the reason for amino acid retention. Although looking more like a cation exchange mechanism, the graphs for the log-log-plots in Figure 5 are still slightly curved, which should not be observed for a pure ion exchange mechanism. To explain this, another variation in eluent composition must be accomplished. The pH of 4.00 was assumed to be too

close to the pI's of the amino acids. Therefore, they do not only experience cation exchange, but a mixed mechanism resulting from cation exchange as well as from changing amino acid charge. The charges of the amino acids do change with eluent ionic strength comparable to the behaviour described for the sodium acetate eluent before. To verify this, separations are repeated using an eluent at a lower pH. Sodium acetate eluents can not be used at a pH much lower than 4.00. Under these conditions an acetate eluent would be present mainly as acetic acid. This would reduce total ionic strength dramatically. Therefore, nitric acid is used to perform separations at a lower pH. The pH of a nitric acid eluent at an ionic strength of 10 mM is far below 4.00. At this pH, a change in nitric acid concentration should not result in a change in the netto charges of the amino acids. The log-log-plot for changing eluent ionic strength of a nitric acid eluent using sulfobetaine C3 is shown in Figure 7. Again, results for the other sulfobetaine exchangers are comparable.

The decrease in retention factors with increasing eluent ionic strength can easily be seen. Contrary to the results found using sodium acetate eluents, the nitric acid eluent gives totally linear relations for all of the analytes. In addition to this, different slopes can be observed for different groups of amino acids. While the slopes for l-leucine, dl-valine and dl-alanine are almost identical, slopes for l-arginine and l-lysine are exactly twice as steep. In ion exchange theory, slopes in these log-log-plots correlate with the charge-to-charge ratio between eluent ions and analyte ions. A doubling in slope, therefore, must correlate with a doubling in charge of the amino acids. According to this observation, l-arginine and l-lysine present the basic amino acids among the examined analytes, carrying two amines instead of one amine for the other amino acids. Therefore, the steeper slope for these analytes reflects the higher charge, and by this, the estimated behavior for cation exchange chromatography. This observation gives the final confirmation for the presence of cation exchange leading to the shown separations.

Besides electrostatic interactions, hydrophobic interactions seem to play a role in amino acid separation. Regarding the elution of already-mentioned amino acids (dl-alanine, dl-valine, and l-leucine), it can be shown that hydrophobicity of the analytes has an influence on retention order. These three amino acids form a homologous row, only differing in the length of the alkyl side chain increasing from methyl to isopropyl and isobutyl. pK_a values for the carboxyl and amino groups, as well as pI values, are almost identical.

If retention would only be based on electrostatic interactions, size should be the crucial property of the analytes. In this case, the largest molecule, l-leucine, should elute first, followed by its smaller homologues. Obviously, retention order is inverted. Therefore, hydrophobic interactions, which are presumably secondary interactions with the un-polar core material, coexist with electrostatic interactions. However, electrostatic interactions are the dominant interactions concerning the dependence of retention times on eluent ionic strength. Elution order does not change when eluent ionic strength is varied, so hydrophobic interactions are constant and independent from eluent ionic strength.

By optimizing the eluent composition, good separations with good peak shapes can be observed using the prepared zwitterionic stationary phases as shown exemplarily for sulfobetaine C3 in Figure 8. In this figure, the baseline separation of eight arbitrarily chosen amino acids is shown. Further improvements are possible by optimizing exchange capacity.

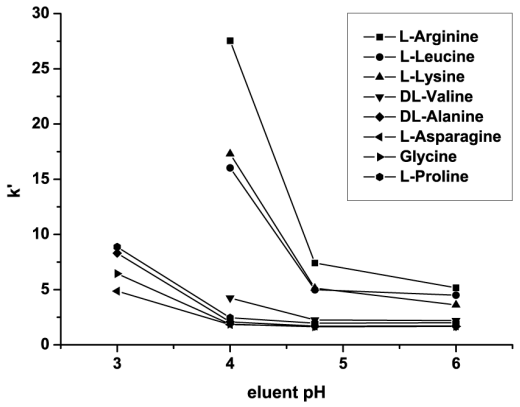

Figure 6. Influence of eluent pH on amino acid retention. Eluent: 20 mM sodium acetate; column: Sulfobetaine C3, PAD; flow rate: 1.0 mL/min; temperature: 308 K.

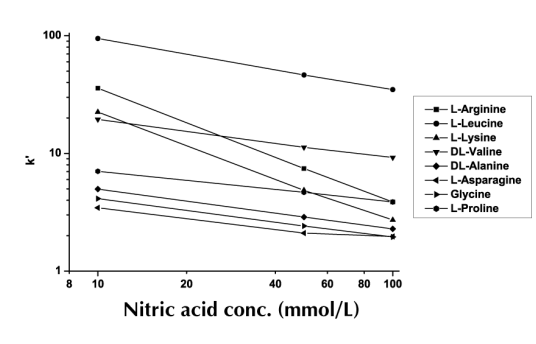

Figure 7. Influence of eluent ionic strength on amino acid retention. Eluent: nitric acid; column: Sulfobetaine C3, PAD; flow rate: 1.0 mL/min; temperature: 308 K.

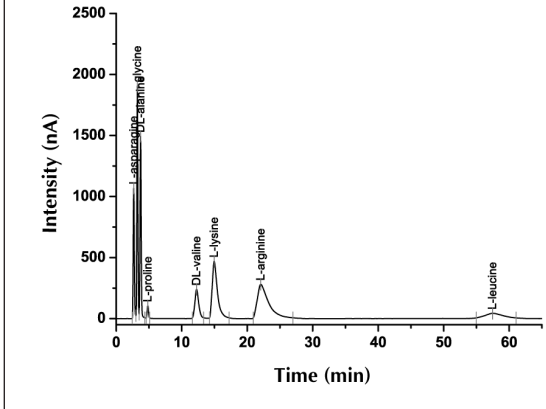

Figure 8. Separation of eight arbitrarily chosen amino acids. Eluent: 10 mM nitric acid; column: Sulfobetaine C3, PAD; flow rate: 1.0 mL/min; temperature: 308 K.

Conclusion

In conclusion, it could be shown and proved by multiple experiments and observations that cation exchange is the dominant separation mechanism for amino acid separations under ZIC conditions using eluents containing eluent ions. These results were found for the whole homologous row of sulfobetaine exchangers as well as for the commercially available ZIC–pHILIC material. It is remarkable that sulfobetaine type exchangers, which up to now have only been known to provide good anion separations, are very well suited for cation exchange separations of α-amino acids. In addition to this, the used core material was found to have a big influence on separations, as seen in the completely different behavior of the ZIC–HILIC material. The baseline separation of eight α-amino acids was achieved.

Acknowledgements

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