



Effect of salts on retention in hydrophilic interaction chromatography[☆]

Andrew J. Alpert

PolyLC Inc., 9151 Rumsey Road, ste. 180, Columbia, MD 21045, USA



ARTICLE INFO

Article history:

Received 17 October 2017

Received in revised form 8 January 2018

Accepted 17 January 2018

Available online 31 January 2018

Keywords:

Hydrophilic interaction chromatography (HILIC)

Electrostatic repulsion-hydrophilic interaction chromatography (ERLIC)

Hydration number

Chaotrope

Kosmotrope

Counterion

ABSTRACT

There is a widespread belief that salts promote retention of solutes in hydrophilic interaction chromatography (HILIC) by expanding the volume of the immobilized layer of water on the surface of the stationary phase. To date, all studies of this premise have had flaws or limitations that left the question open. This study explored the effects of salt type and concentration.

The effect of the anion was studied with four triethylammonium salts, ranging from the kosmotropic sulfate to the chaotropic perchlorate, at pH values of both 3 and 6. Concentrations ranged from 5–120 mM. All analytes were neutral except for cytosine and cytidine, which had (+) charge at pH 3. Sulfate markedly promoted retention of cytosine, cytidine and phloroglucinol. At high sulfate levels retention of cytosine and cytidine decreased again, presumably due to a “salting-out” effect. With perchlorate anion, retention of cytosine decreased steadily as salt concentration increased, while retention of other standards increased or was unchanged.

The effect of the cation was examined by comparing the retention of a tryptic peptide containing either phosphoserine or aspartic acid at the same position. Salts of methylphosphonic acid were used at pH 2.5. The higher the hydration number of the cation, the better the selectivity between the two peptides. The best separation was obtained with the magnesium salt and the worst with the tetramethylammonium salt. The retention contributed by a highly hydrated cation exceeded retention due to electrostatic attraction.

These results demonstrate that counterions that are well hydrated serve to promote partitioning of charged solutes into the immobilized aqueous layer in HILIC, while poorly hydrated counterions have the opposite effect. Effects on neutral solutes were more modest; retention times remained unchanged or increased modestly with an increase in concentration of any salt.

© 2018 The Author(s). Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).

1. Introduction

HILIC was introduced as a general-purpose mode for separation of polar solutes in 1990 [1] and is now in widespread use. The mechanisms involved in HILIC separations appear to be complex and have been the subject of a number of studies. Initial speculation [1] involved a semi-immobilized layer of water associated with the polar stationary phase, with polar solutes partitioning between this layer and the more dynamic, predominantly organic bulk of the mobile phase. In 2006, Hemström and Irgum examined this scenario and concluded that the evidence tended to support it [2].

Mountain [3] and Melnikov *et al.* [4] have subsequently obtained spectroscopic evidence of a diffuse layer of immobilized water about 11 Å thick and a more rigid layer of water about 4 Å thick in the immediate vicinity of the stationary phase surface. Other forces that may be involved include ion-exchange effects, either attractive or repulsive [5], and hydrogen-bonding [6].

In the past few years, a number of papers have appeared in the literature that presume that increasing the salt concentration results in increased retention in HILIC. The source of this belief seems to be a paper from 2005 by Guo and Gaiki [7], in which increasing concentrations of ammonium acetate were associated with increasing retention of several solutes. While the authors did acknowledge the possibility of the shielding of electrostatic repulsion, they also speculated about the expansion of the aqueous phase effected by the hydration of the salt ions, with a consequent increase in retention of polar analytes. In a subsequent paper these authors put more emphasis on the shielding of charged ana-

[☆] Selected paper from 45th International Symposium on High Performance Liquid Phase Separations and Related Techniques (HPLC 2017), 18–22 June 2017, Prague, Czechia.

E-mail address: aalpert@polylc.com

lytes from electrostatic repulsion by the stationary phases, many of which have some degree of negative charge under the conditions used for HILIC [8]. They have also commented on the lack of evidence for the effect of salt on the structure or volume of the immobilized aqueous layer [9]. Despite this absence of evidence, the original speculation from 2005 continues to be cited widely.

There are a number of papers in the literature with systematic investigations of the effects of salt on retention in HILIC. Most of them have suffered from one of the following limitations:

- 1) The standards used were charged. That made it difficult to separate the effect of salt on electrostatic attraction/repulsion from effects on partitioning into the immobilized aqueous layer.
- 2) The investigation involved only a single compound.
- 3) Only one salt was studied. With few exceptions, it was a salt that happened to be convenient for their detection method, e.g., ammonium acetate or formate.
- 4) The wrong salt was studied or a helpful salt was avoided. Some studies have expressly avoided phosphates or sulfates out of concern that all of them would have limited solubility.
- 5) Study of a limited range of salt concentration. Again, with few exceptions, it was a range compatible with their intended detection method: 2–20 mM. The problem is that such concentrations are too low to titrate all charged groups in most stationary phases; that requires up to 30–40 mM salt even with a “neutral” material [5,8,9]. Such studies have chosen convenience rather than control of a variable that could shed some light on the mechanism.

The present study has been designed to try to avoid the limitations listed above, and hopefully elucidate the effect of added salt on the structure and properties of the immobilized aqueous layer in HILIC. In the study of the effect of the anion, the standards were neutral (Fig. 1) with the exception of cytosine and cytidine, which are positively charged at pH 3. They were included anyway because of their use as probes of polarity in studies on the properties of materials used for HILIC [10]. The overall salt concentration in the mobile phase was varied between 5 and 120 mM. The column used was PolyHYDROXYETHYL A. This is a silica-based material with a thick, covalently attached coating of a neutral, hydrophilic polypeptide [1]. As with any silica-based material, there is a low level of electrostatic charge. It has a slight positive charge below pH 4.4 and a slight negative charge above it, while at pH 4.4 it is in a zwitterionic balance and is truly neutral [11]. 20 mM salt suffices to titrate the charged residues (ref. [8], Fig. 7). This is less than is needed to eliminate electrostatic interactions with other neutral HILIC stationary phases (ref. [8], Figs. 6 and 7). It also features the thickest immobilized aqueous layer of any neutral stationary phase investigated to date [12,13].

The most important variable was the selection of the salts. In order to properly assess the role of hydrogen bonding and other dipole–dipole interactions, salts were selected that either promoted or antagonized these to varying degrees. Salts that promote hydrogen bonding, called kosmotropes, are those that are high in the Hofmeister series [14]. Examples include citrates, tartrates, sulfates, and phosphates. Kosmotropic ions have thick, strongly held spheres of hydration [15,16]. A high concentration of a kosmotropic salt in solution, such as ammonium sulfate, can deny a protein sufficient water to form a sphere of hydration. The protein may then self-associate as a separate phase and precipitate, a process called “salting-out”. Salts that antagonize hydrogen bonding are chaotropes. These have thin, weakly-held spheres of hydration. Examples include perchlorates, trifluoroacetates, iodides, and unbuffered acids. In general retention is less with trifluoroacetate ion [17,18] than with acetate or formate salts. There is some confusion in the literature as to the nature of the effect of both kos-

motropes and chaotropes on HILIC. Bicker *et al.* [17] described the chaotropic trifluoroacetate as a more “lipophilic” ion than formate or acetate and so, to paraphrase, an immobilized aqueous layer containing it would differ less in polarity from the predominantly organic bulk mobile phase than would an aqueous layer containing formate or acetate [NOTE: They appear to assume that the trifluoroacetate ion remains resident in the stagnant aqueous layer. This assumption is examined here in Discussion]. Kamichatani *et al.* [18] cited references to the effect that ion-exchange resins swell in the presence of kosmotropes and shrink in the presence of chaotropes. If a weakly-hydrated chaotropic ion associates with a stationary phase, then that should reduce the thickness of the hydration layer in HILIC. This scenario assumes that the stationary phase is charged and the association is through electrostatic attraction. In fact, Kamichatani *et al.* have the situation backwards. Chaotropic salts cause a zwitterionic polymer to swell to a much greater degree than do kosmotropic salts [19]. This is true as well with neutral, well-hydrated polymers. The coating of PolyHYDROXYETHYL A does swell appreciably in the presence of a chaotrope, presumably because the chaotrope disrupts the hydrogen bonds between adjacent chains in the coating [20].

In the last few decades, the Hofmeister classifications have been updated with the characterization of ions in terms of specific physical properties. These include the chemical potential or Gibbs free energy for partitioning between separate phases, various colligative properties [20], and hydration under various conditions [21,22]. For purposes of the current study, a particularly useful property is the degree of hydration of an ion upon its transfer from water to an immiscible organic solvent [23]. For this study, salts were selected with the following anions: Sulfate, a strong kosmotrope; formate, a weak kosmotrope; bromide, a weak chaotrope; and perchlorate, a strong chaotrope. While no single study in the literature includes all four of these ions, overlapping lists indicate that their degrees of hydration decrease more or less in the order listed. Their triethylammonium salts were prepared by addition of triethylamine to aqueous solutions of the acids. Retention is weaker in HILIC with triethylammonium salts when compared with the corresponding ammonium salts [17], but this use of an organic cation permitted a high concentration of anions such as sulfate to be maintained in a predominantly organic mobile phase. This factor was considered to outweigh the importance of choosing a more hydrophilic cation. It should also be noted that perchlorate and bromide ion have virtually no buffering power at pH 3 and so there is practically no difference in the composition of the mobile phases containing these ions at pH 3 and pH 6. It was judged that consistency in the composition of the anion outweighed this factor as well. A final compromise was that all solutions were made up at the same molarity even though the normality of sulfuric acid is twice that of the other acids.

The effect of the cation was studied using various metal salts of methylphosphonic acid as additives. The retention of two peptides was compared, one with a phosphoserine residue and the other with aspartic acid at the same position. The pH of 2.5 was low enough for the carboxyl- group of the aspartyl- residue to be substantially uncharged. The results obtained with a HILIC column were compared with those obtained using an anion-exchange column in order to distinguish the effects of hydrophilic interaction from electrostatic effects.

2. Materials and methods

2.1. Column

For HILIC of small molecules a column of PolyHYDROXYETHYL A was used based on 3- μm , 100- \AA silica (PolyLC Inc., Columbia, MD, USA; item 104HY0301). For HILIC of peptides a column of the same

type was used with a pore diameter of 300 Å (item 104HY0303). The anion-exchange column used for peptides was a PolyWAX LP column based on 3- μ m, 300-Å silica (PolyLC Inc.; item 104WX0303). All columns were 100 \times 4.6-mm.

2.2. Reagents

Acetonitrile (ACN) was HPLC-grade. All acids were ACS-grade. Formic acid (98%) was from EMD (Billerica, MA). Cyclo(Ala-Gly) and cyclo(Ser-Ser) were from Research Plus (Bayonne, NJ). All other standards and reagents were from Sigma-Aldrich (St. Louis, MO). These included sulfuric acid (95–98%), perchloric acid (60%), hydrobromic acid (48%), triethylamine ($\geq 99.5\%$), methylphosphonic acid (item# 289868; 98%), sodium hydroxide (ACS; $\geq 97\%$), lithium hydroxide monohydrate (BioUltra; $\geq 99.0\%$), cesium hydroxide (50 wt.% solution in water; 99.9%), magnesium hydroxide (BioUltra; $\geq 99.0\%$), ammonium hydroxide (28% NH₃ in water; $\geq 99.99\%$), and tetramethylammonium hydroxide pentahydrate ($\geq 97\%$).

The peptides WWGSGPSGGDGGGK (P1) and WWGSGPS-GSGG(pSer)GGGK (P2) were synthesized by United Biosystems (Herndon, VA).

2.3. Methods

Triethylammonium salts: Stock solutions of the salts were prepared by adding triethylamine (TEA) to aqueous solutions of the acids, with stirring, until reaching pH 3 or 6. The stock solutions were 1 M in terms of the anion. They were filtered through a 0.45- μ m nylon mobile phase filter. Mobile phases were prepared by adding measured amounts of the stock solution and water to a graduated cylinder, then adding ACN to within several ml of the calibration mark followed by inversion 8–9 \times . Once the contents had warmed up to room temperature, ACN was then added to the level of the mark. An ACN concentration of 85% was used throughout. The pH was measured only of the stock solutions, not the mobile phases. It was not possible to prepare a mobile phase solution containing 120 mM TEA-sulfate, pH 6; phase separation resulted. Salt concentrations are given for the mobile phase overall (*i.e.*, after addition of the ACN).

2.3.1. Methylphosphonate salts

These were prepared similarly, adding the metal hydroxide to an aqueous solution of methylphosphonic acid until reaching pH 2.5. Again, molarity was in terms of the anion.

Detection was via absorbance at 225 nm. When formate was used, the elevation in the baseline was zeroed out. This was not practical with the higher concentrations of bromide, which has a molar extinction coefficient approximately twice that of formate at 225 nm. Accordingly, mobile phases containing bromide were monitored at 230 nm in order to stay within the linear response range of the detector.

The column was equilibrated with each new mobile phase for one hour before samples were run. Elution was isocratic, at 1 ml/min. This equilibration was more prolonged than is usual in HILIC, to insure complete replacement of the surface counterion layer. Each standard was run both individually and in a mixture with every mobile phase. Some standards required about 10% more water than was in the mobile phase in order to attain a reasonable concentration in solution. Analyses were at ambient temperature.

3. Results

3.1. Effect of varying the anion

Fig. 1 compares the elution of the standards with 5–120 mM TEA-sulfate (pH 3) in the mobile phase. The retention of cytosine

and cytidine is markedly greater, relative to the other standards, than is the case in other studies in the literature that involve less kosmotropic anions. Two trends in particular are noteworthy: a) Retention of cytosine and cytidine increases steadily between 5 and 40 mM, then decreases significantly as the salt concentration increases to 80 and 120 mM; b) Except at 120 mM salt, where they coelute, cytosine elutes later than does the nucleoside cytidine. This may be the first reported instance where addition of a neutral sugar residue to a solute decreases its retention in HILIC. A modest decrease in the retention of guanosine is also evident with 120 mM sulfate.

Another unusual trend is the appreciable increase in retention of phloroglucinol with sulfate concentration, to an extent that is anomalous when compared with the other neutral standards. Phloroglucinol is not charged, and this increase is evident at both pH 3 and 6. A much more muted version of this trend is seen with the weaker kosmotropic anion formate, and it is absent with the chaotropic anions bromide and perchlorate. The basis for this behavior by phloroglucinol is obscure.

Fig. S1 shows the sulfate retention data graphically at pH 3 while Fig. S2 shows it at pH 6. The data at pH 6 only runs through 80 mM because 120 mM afforded phase separation. Retention of cytosine and cytidine are considerably lower at pH 6, where they are not charged and so have no counterion, than was true at pH 3.

Fig. 2 compares the elution of the standards with 5–120 mM TEA-perchlorate (pH 6). Retention of analytes either increases modestly with salt concentration, guanosine in particular, or remains unchanged. Again, cytosine was an exception, exhibiting a modest but steady decrease in retention as salt concentration increased. Retention of cytidine decreased modestly up to 40 mM and increased modestly thereafter. Both cytosine and cytidine were far less well retained with this salt than with TEA-sulfate at pH 3. Retention of the other solutes was not markedly different. Figs. S3 and S4 show the retention data with perchlorate graphically at pH 3 and 6, resp.

Figs. S5 and S6 show the retention data with formate graphically at pH 3 and 6, resp. The trends at pH 3 resemble a muted version of those with sulfate at pH 3, with moderate increases in the retention of cytosine and cytidine with salt concentration and a modest decrease with 120 mM in their retention and in the retention of guanosine. Figs. S7 and S8 show similar graphs for retention with bromide. A change in TEA-bromide concentration results in no noteworthy changes in retention of the standards.

Figs. S9–S16 are graphs of the retention of individual standards throughout the concentration range of all salts.

In Fig. 3, retention is compared with a 40 mM concentration of all salts at pH 3. With sulfate, the marked disparity between the retention of cytosine and cytidine and of the other analytes is readily apparent. This is also true for phloroglucinol. It is evident that the positive charge alone does not account for the disparate behavior of cytosine and cytidine at pH 3; the properties of the counterion involved are critical. This same disparate behavior is also evident in Fig. 4 at pH 6 (80 mM salt concentration), to a lesser extent for cytosine and cytidine (which are uncharged at this pH) but to the same extent for phloroglucinol.

Some trends are evident from this data. Guanosine, uridine, cyclo(Ala-Gly) and cyclo(Ser-Ser) are less sensitive to changes in the salt and its pH and concentration than are cytosine, cytidine, and phloroglucinol. There is a notable increase in retention of guanosine with sulfate concentration up to 40 mM. At 120 mM, pH 3, its retention begins to decrease, as with cytosine and cytidine. With sulfate at pH 6, retention of guanosine started lower than at pH 3 but by 80 mM the two curves for sulfate were converging. The phase separation occurring with 120 mM sulfate, pH 6, prevented this data point from being collected.

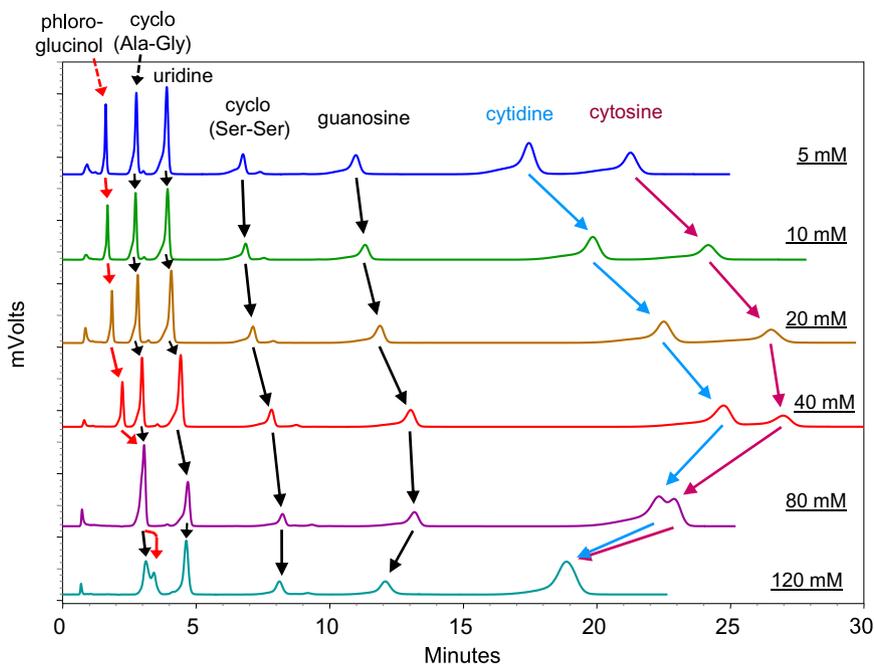


Fig. 1. HILIC of standards at various concentrations of TEA-sulfate. Column: PolyHYDROXYETHYL A (100-Å pore). Mobile phase: 85% ACN with TEA-SO₄, pH 3 (concentration as noted). Flow rate: 1.0/min. Detection: 225 nm.

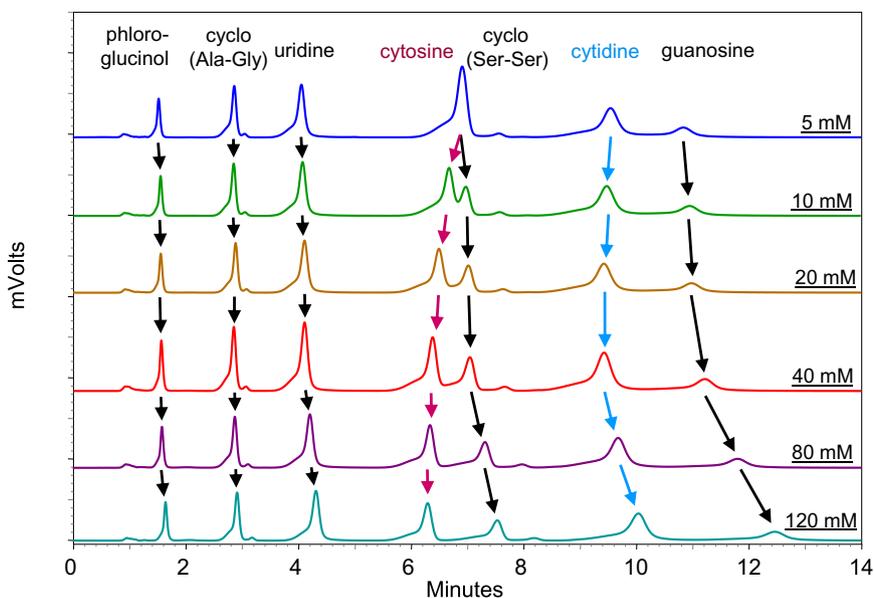


Fig. 2. HILIC of standards at various concentrations of TEA-ClO₄. Column: As per Fig. 1. Mobile phase: 85% ACN with TEA-ClO₄, pH 6 (concentration as noted).

3.2. Effect of varying the cation

When cations partition from water into an immiscible solvent, they are accompanied by the following numbers of water molecules (Osakai et al. [23]): Ca⁺² (14); Li⁺ (6.0); Na⁺ (3.8); Cs⁺ (0.4); (CH₃)₄N⁺ (0). These ions, as well as NH₄⁺ (with hydration similar to that of Me₄N⁺ [21]) and Mg⁺² (more highly hydrated than Ca⁺² [20,21,22]), were compared for their effect on the retention of a phosphopeptide in HILIC.

The peptide standards peaks exhibited fronting with the 100-Å column. This was evidently due to steric hindrance, since it disappeared with a 300-Å column (Fig. 5). In retrospect, that may account for some of the fronting seen with the small molecules in the study of the effect of the anion; the PolyHYDROXYETHYL A coating is thick

enough to partially fill in a 100-Å pore under some conditions. The surface area of the 300-Å material was lower and so retention times were shorter, but were still more than adequate.

Fig. 6 shows the isocratic separation of peptide P1 from the phosphopeptide P2 with 75% ACN. The minor peak eluting first was a byproduct from the synthesis of P1. It proved to be the same peptide but with a succinimide ring [Asu] (a dehydration product) in place of the aspartyl- residue. The separation of this byproduct from P1 suggests that there is a small degree of residual charge on the aspartyl- sidechain at pH 2.5. With monovalent metal cations, both the retention of P2 and its separation from P1 increased in proportion to the degree of hydration of the cation. With Mg⁺², which is significantly more highly hydrated than the monovalent cations, this separation was dramatically greater. Ion pairing with

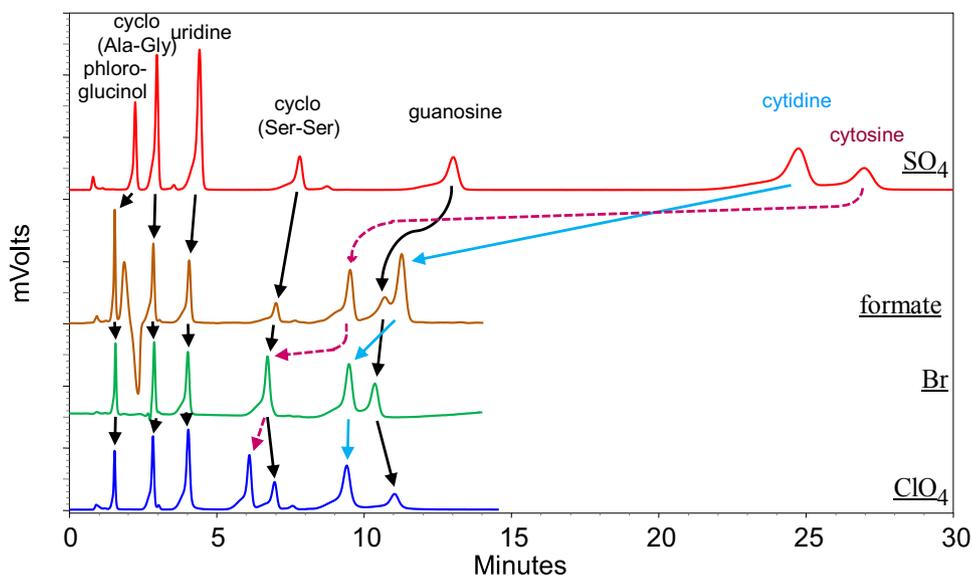


Fig. 3. HILIC of standards with 40 mM TEA salts, pH 3, and 85% ACN. Column and conditions: As per Fig. 1.

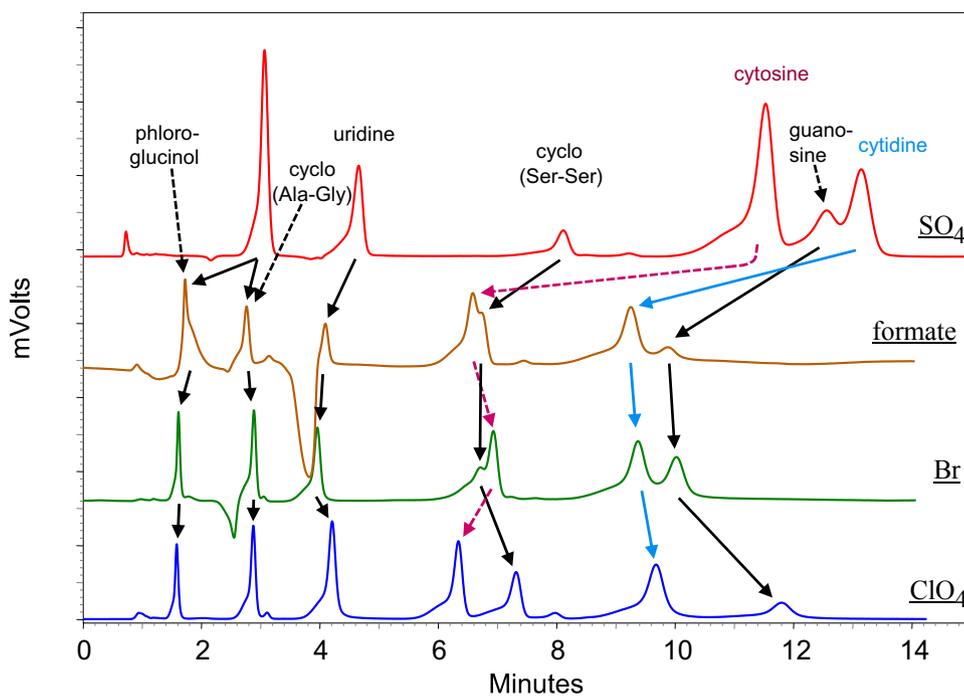


Fig. 4. HILIC of standards with 80 mM TEA salts, pH 6, and 85% ACN. Column and conditions: As per Fig. 1.

the $(\text{CH}_3)_4\text{N}^+$ ion, which has a degree of hydration ~ 0 , caused the phosphopeptide to elute earlier than the nonphosphopeptide. With 70% ACN hydrophilic interaction was tuned down but not electrostatic effects, leading to lower retention overall and less clearcut effects of ion hydration. The more hydrated ions still afforded better selectivity between P1 and P2 but not necessarily stronger retention.

Some results were obtained with the methylphosphonate salt of Ca^{+2} . They were similar to those obtained with Mg^{+2} albeit with somewhat shorter retention times for the phosphopeptide. This is consistent with the lower degree of hydration of Ca^{+2} compared with Mg^{+2} . Its solubility in the mobile phase was marginal, though, and the lines clogged repeatedly with precipitate before a complete data set was obtained, so none is shown for Ca^{+2} . The methylphosphonate salts of Zn^{+2} , Al^{+3} and Ce^{+3} were even less soluble in 70% ACN and no data was obtained with them.

Use of an anion-exchange column instead of a neutral column introduces electrostatic repulsion of most peptides at the low pH used here, since they have net (+) charge. In the presence of >60% ACN, this combination is called electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) [5]. Phosphate, sulfate or sialyl- residues maintain some (-) charge at this pH and experience electrostatic attraction. Fig. 7 compares retention between HILIC and ERLIC conditions. Retention of the unphosphorylated peptide P1 decreases, while retention of the phosphopeptide increases with monovalent cations. The result with Mg^{+2} is a marked exception; there is practically no difference between the two columns, and the phosphopeptide actually elutes slightly earlier from the anion-exchange column than from the HILIC column. Evidently the gain in retention from hydrophilic interaction with so highly hydrated a counterion outweighs any gain from electrostatic attraction, at least with 75% ACN present, and the two effects are not additive.

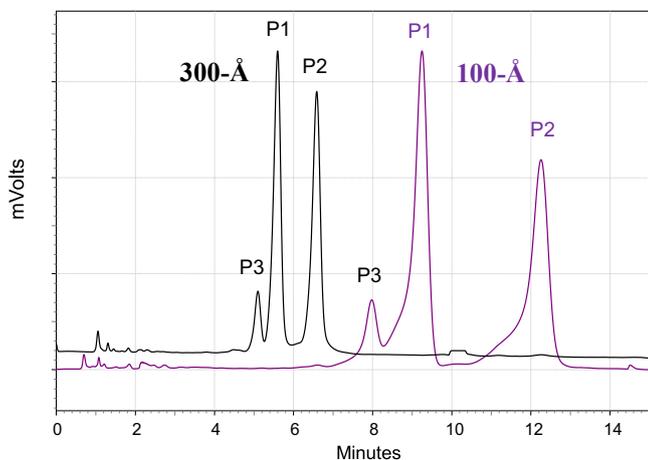


Fig. 5. HILIC of peptides on PolyHYDROXYETHYL A columns with either 100-Å [purple trace] or 300-Å [black trace] pores. Mobile phase: 70% ACN with 20 mM sodium methylphosphonate, pH 2.5. Flow rate: 1 ml/min. Detection: 225 nm. Peptide standards: WWGSGPSGSGDGGGK (P1), WWGSGPSGSGG(pS)GGGK (P2), and WWGSGPSGSGG(Asu)GGGK (P3). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

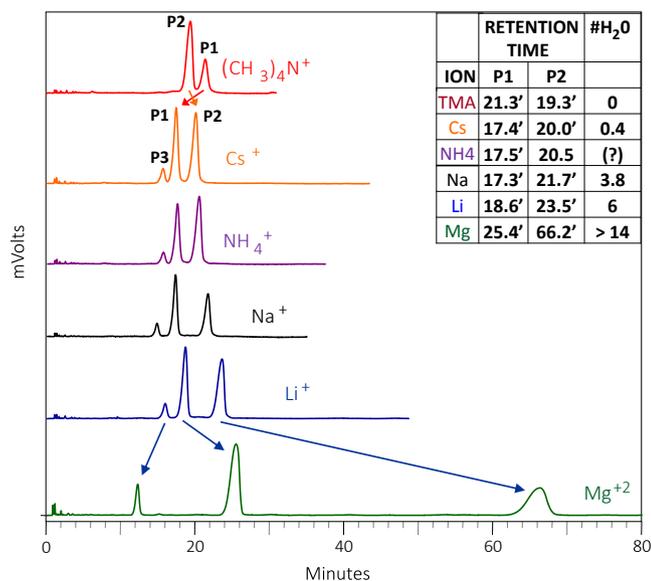


Fig. 6. HILIC of peptides with various cations. Column: PolyHYDROXYETHYL A, 300-Å pores. Mobile phase: 75% ACN with 20 mM of the methylphosphonate salt indicated, pH 2.5. Other conditions and standards as per Fig. 5. Inset: Figures for hydration numbers are from ref. [23].

Fig. 8 presents the results obtained in ERLIC with 70 and 75% ACN. With 70% ACN the addition of electrostatic effects results in deviation from the elution orders observed with HILIC. Peptides P1 and P2 now elute in the same sequence with all cations, including $(\text{CH}_3)_4\text{N}^+$, but the relative retention times do not correlate with the hydration of the cations. With 75% ACN the hydrophilic interaction is stronger and retention times do correlate with the hydration of the cations.

4. Discussion

Ibrahim and Lucy have reported on the retention of anions on an anion-exchange monolith in the HILIC mode [24]. Chaotropic anions exhibited the strongest electrostatic attraction but the weakest hydrophilic interaction. Retention of all anions decreased with increasing ACN concentration. Above 80% ACN retention of kosmotropic anions began to increase again as their hydrophilic

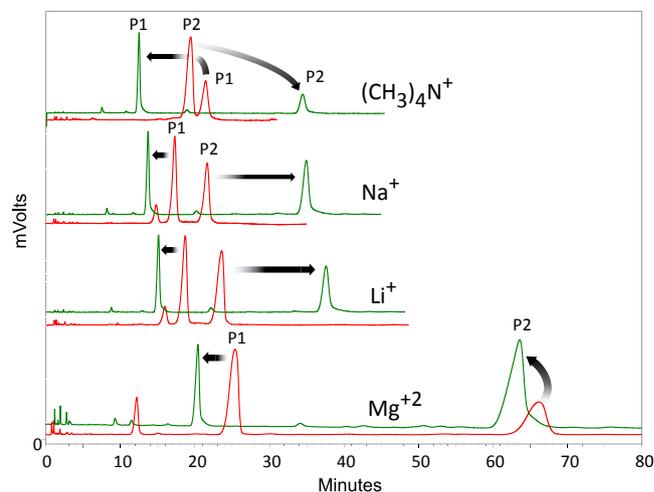


Fig. 7. HILIC and ERLIC of peptides with various cations. HILIC (red traces): PolyHYDROXYETHYL A column, 300-Å pores. ERLIC (green traces): PolyWAX LP column, 300-Å pores. Mobile phase: 75% ACN with 20 mM of the methylphosphonate salt indicated, pH 2.5. Other conditions and standards as per Fig. 5. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

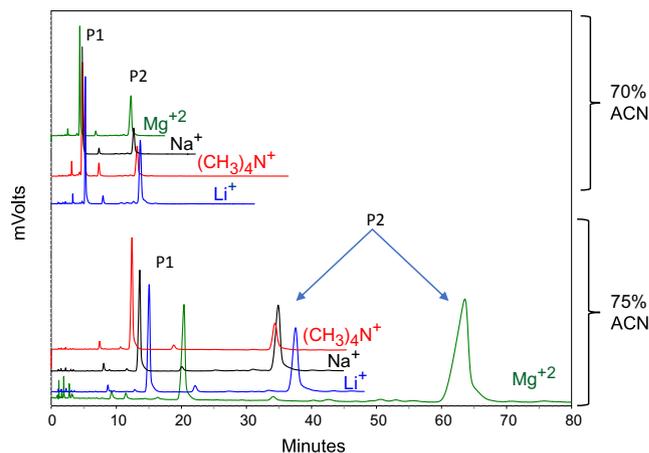


Fig. 8. ERLIC of peptides with various cations and ACN concentrations. Column, conditions and standards: As per Fig. 7, except that the ACN concentration was either 70% [TOP] or 75% [BOTTOM].

interaction became more prominent but the retention of chaotropic anions (iodide; nitrate) continued to decline almost to zero. This demonstrates that chaotropic ions, which are poorly hydrated, tend to partition into the predominantly organic mobile phase in HILIC. This evidence contradicts the evident assumption of Bicker et al. [17] that “lipophilic” ions such as trifluoroacetate remain in the stagnant aqueous layer and so lower the difference in polarity between it and the predominantly organic mobile phase. These observations also account for some of the data here regarding cytosine and cytidine. At pH 3 they form ion pairs with the kosmotropic (and well-hydrated) anion sulfate. This promotes their partitioning into the stagnant aqueous phase rather than the dynamic, predominantly organic mobile phase. The same is true of the weakly kosmotropic anion formate to a lesser extent, at least regarding cytosine. The retention of cytosine and cytidine is less distinctive at pH 6, where they are uncharged and so have no counterion. Formation of ion pairs with the poorly hydrated perchlorate anion does not increase retention, at least with 85% ACN, and actually decreases retention to some extent, consistent with Ibrahim and Lucy’s data. The results with various cations were also consistent with this. With a high level of hydrophilic interaction, at 75% ACN,

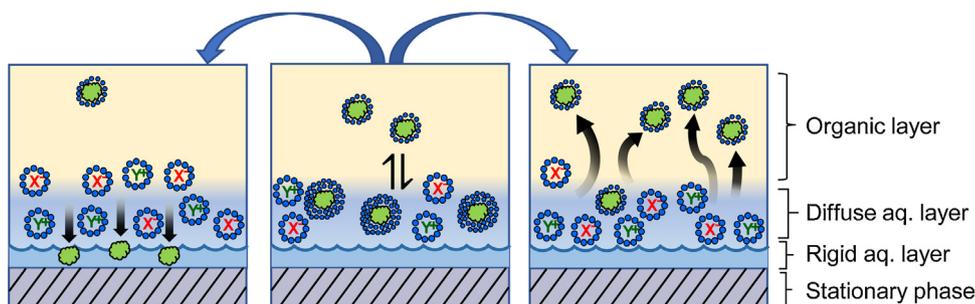


Fig. 9. Schematic of proposed alternative effects of added salt in HILIC. [RIGHT] Driving solutes into the predominantly organic phase via a conventional salting-out mechanism (this paper). [LEFT] Enhanced partitioning into the rigid aqueous layer on the surface (“salting-out” per Jandera et al. [25]). Green shapes represent solutes, with blue balls denoting various degrees of hydration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

retention of a phosphopeptide was proportional to the hydration of the cation counterion in either the HILIC or ERLIC mode.

This data makes plain that it is simplistic to state that increasing salt concentration results in increasing retention in HILIC. Depending on the solute and the salt, in some cases retention is in fact increased while in others it is decreased. The results with perchlorate also suggest that a swelling of the stationary phase coating does not necessarily lead to a significant increase in retention. A description of “hydrophilic interaction” needs to take the following properties and mechanisms into account:

4.1. Different effects with low, medium, and high salt concentrations

The trends apparent in this data seem to fall into two different categories depending on whether the salt concentration is <30 mM or >30 mM. At low concentrations, added salt will plausibly serve to provide counterions for charged groups on the stationary phase. This could affect the overall charge of the surface, which would influence the retention of charged solutes more than uncharged ones. Higher concentrations would complete the titration of the surface, with additional salt serving to affect the structure of the bulk of the immobilized water layer. An example is evident in Fig. S2 (TEA-sulfate, pH 6), in which there is generally little change in retention times until 20 mM salt. Between 20 and 40 mM there is an abrupt increase in the retention of some of the standards. The retention of guanosine shows a similar sensitivity to salt concentration in Fig. S6 (TEA-formate, pH 6), decreasing above 20 mM. The trends with concentration of the chaotropes perchlorate and bromide are more gradual. This may be because, being poorly hydrated, they tend to partition less into the immobilized aqueous phase. Electrostatic effects aside, a much higher concentration of a chaotropic salt may be required to get the same concentration of salt into the immobilized aqueous layer than would be needed with a kosmotropic salt. This differential would become more extreme at higher concentrations of organic solvent.

A still different mechanism seem to come into play at the highest concentrations of salt. The term “salting-out” is taken to mean that a high concentration of a kosmotropic salt sequesters the water in its solution, causing other solutes to self-associate (leading to precipitation of proteins) or else to partition into a nonaqueous phase if one is present. This nonaqueous phase can be either a layer of organic solvent or the surface of a stationary phase for hydrophobic interaction chromatography (HIC). In HILIC, one would expect salting-out to take the form of a shift in partition coefficient to favor the mostly organic mobile phase, with a decrease in retention times. Fig. 9 portrays this model and the alternative model of Jandera. Jandera has described salting-out in terms of the salt promoting increasing association with uncharged stationary phases through formation of hydrogen bonds, thereby increasing retention [25]. It is possible that this is true at concentrations of salt

<20 mM. However, with the highest concentrations of kosmotropic salt used in the present study (80 and 120 mM TEA-sulfate (pH 3; Figs 1 and S1) and 120 mM TEA-formate (pH 3, Fig. S5), one sees the decrease in retention that one would expect with salting-out, with some solutes partitioning into the predominantly organic mobile phase as it becomes increasingly difficult for them to remain solvated in the immobilized aqueous layer. The same trends have been observed with kosmotropic salts in addition to sulfates. Phosphate is comparable to sulfate in its kosmotropic properties and degree of hydration. When nucleotides are run on a cation-exchange column in the HILIC mode, there is a steady increase in retention up to 40 mM TEA-phosphate as the electrostatic repulsion is shielded [5]. Between 40–120 mM, retention decreases again. Presumably this reflects salting-out. The same effects are noted with basic amino acids run in the HILIC mode on an anion-exchange column [5] and with nucleic acid bases run on an anion-exchange column [26]. It is plausible that in the range 40–120 mM salt, solutes such as cytosine, cytidine, and guanosine are associated with the diffuse aqueous layer and that the high concentrations of kosmotropic salt antagonize their ability to remain hydrated in it. Solutes whose retention is not affected by the highest concentration of TEA-sulfate or TEA-formate, such as the cyclopeptides and uridine, may be associated with the more rigid aqueous layer on the surface of the stationary phase. It is also possible that the high concentrations of salt are in fact driving the association of phloroglucinol with that rigid aqueous layer per the model proposed by Jandera.

4.2. Chaotropic vs. kosmotropic salts

Chaotropes disrupt hydrogen bonds. If an analyte is retained in HILIC through hydrogen bonds, then one would expect its retention to decrease as the concentration of TEA-perchlorate increases. That was the case with cytosine. It was also true of cytidine below 40 mM TEA-perchlorate but not at higher concentrations. It was also not true of the other standards here; their retention tended to increase at high TEA-perchlorate concentration. These observations imply that retention in HILIC does not always involve a significant contribution from hydrogen bonding. The retention of cytosine and cytidine probably involves varying contributions from several mechanisms. Specifically, it is plausible that the retention of cytosine does involve hydrogen bonding to a substantial extent. Dinh et al. [10] used the adenosine/adenine pair to probe hydrogen bonding properties of HILIC stationary phases. Their results suggested that hydrogen bonding with the stationary phase involves a high degree of orientation. Applying that reasoning to the present results, it is possible that the strong retention of cytosine sulfate involves hydrogen bonding through position 1 of the cytosine ring. Attachment of the ribose residue at that position could have disrupted the orientation of the molecule and antagonized the hydrogen bonding, resulting in a decrease in retention that was more significant than any increase in hydrophilic interaction con-

ferred by the ribose. With sulfate at pH 6, cytosine and cytidine are neutral and this rigid orientation would not pertain. Their retention is then more in line with that of the other standards (although still greater than with the other salts in the mobile phase), and elution is in the conventional sequence cytosine-cytidine. That is also true at pH 3 with the other anions; any rigid orientation of cytosine and cytidine must be a property of the sulfate salts only (or kosmotropic salts only; results with formate were similar but dampened compared with sulfate) and not just due to their possessing positive charge.

In addition to the anomalous behavior observed here when compared with other standards, cytosine is reported to behave anomalously in HILIC at pH 7 when various salts are used for elution [18]. A number of papers have commented on its charge at low pH. For this reason, Kawachi et al. [27] have recommended the use of guanosine or uridine derivatives as standards for probing the properties of chromatography materials. The present results tend to support this recommendation, since guanosine and uridine were much less affected by the choice of anion and pH than were cytosine and cytidine.

4.3. The effect of the electrical double layer on the surface

An increasing concentration of salt contributes counterions that titrate charged sites on the stationary phase surface. The resulting electrical double layer is complete by a concentration around 20–30 mM for most materials used for HILIC. A layer of a multivalent anion such as sulfate or phosphate could change the effective surface charge from positive to negative [5]. That could account for the increase in retention of cytosine and cytidine up to 40 mM sulfate at pH 3, where they have net (+) charge. However, some increase is also seen over this range with the monovalent anion formate, which would not reverse the net charge on the surface. Concentrations of bromide or perchlorate up to 40 mM either have no effect on retention of cytosine and cytidine or decrease it. Also, increasing concentrations of sulfate at pH 6 promote the retention of several neutral standards as well as cytosine and cytidine (Fig. S2). It is difficult to isolate the effect of the charge of sulfate and phosphate from their properties as strongly kosmotropic, well-hydrated ions.

4.4. Adsorption vs. partitioning

Gritti et al. [28] have proposed a model for retention in HILIC, based on distribution between the rigid and the diffuse water layers described in refs. [3] and [4]. Retention in the rigid water layer is described as “adsorption” while retention in the diffuse water layer is termed “partitioning”. An examination of the behavior of cytosine led the authors to conclude that its retention in HILIC is due more to adsorption than to partitioning. This model discounted electrostatic effects on the basis of two premises: 1) With 10 mM ammonium acetate, pH 5, in the mobile phase, cytosine is neutral; 2) The stationary phase, a BEH material, is also neutral at this pH. One might reasonably be skeptical of both premises. While not as acidic as silica, BEH material does in fact exhibit significant acidic character in this pH range [29]. Secondly, 10 mM is well short of the ~30 mM salt needed to titrate most HILIC stationary phases [5,8,9], including a BEH material, and so shield electrostatic effects. Finally, if the stationary phase is charged, then the microenvironment on the surface may be several pH units above or below the pH in the bulk mobile phase. This is evident in HILIC of phosphopeptides on an anion-exchange material [30]; retention begins to increase as the pH of the mobile phase reaches 4 or above, even though the pKa of the second ionization of the phosphate residues is around 6.8. Accordingly, the behavior of cytosine in the system of Gritti et al. could conceivably be attributable to electrostatic attraction, with

ionization of cytosine being induced by the acidic BEH material. Presumably a test with a range of salt concentration would settle the matter.

Having said that, it is worth considering the possibility that this model does describe the interaction of solutes with the stationary phase at some concentrations of salt. As noted above, at concentrations <30 mM, much of the salt would be forming counterions with charged groups on the surface. The resulting electrical double layer would modify the charge and polarity characteristics of the surface, depending on the counterions involved [5]. It is reasonable that the impact of these changes would be greatest on solutes that partition chiefly into the thin, rigid water layer immediately adjacent to the surface (“adsorption”, per Gritti et al.). No standard in the present study exhibits greater sensitivity to the salt in the 5–30 mM range than does cytosine, whether involving increasing retention (sulfate at pH 3 or 6; formate at pH 3) or decreasing retention (perchlorate at pH 3 or 6). One might speculate that cytosine is “adsorbed” by the rigid water layer through the hydrogen bonding discussed above. That would account for the steady decrease in its retention as perchlorate ion concentration increases. It may even be the case that with the formation of a complete electrical double layer at salt concentrations ≥ 30 mM, no solutes are associated with the rigid aqueous layer and so all hydrophilic interactions involve the diffuse aqueous layer only.

In order to verify or disprove these speculations regarding the forces involved in the retention patterns observed here, it would be helpful to know how the structure of immobilized water layers is affected by various salts over a wide range of concentration. This could presumably be done through the spectroscopic methods of Mountain and of Melnikov et al. [3,4]. The results would more closely relate to HILIC in a column if the silica substrates being studied were derivatized with coatings of the types commonly used for HILIC materials [31].

Acknowledgements

The identification of the succinimide-containing peptide byproduct by MS/MS was performed by Dr. Xiaofang Zhong, in the group of Dr. Lingjun Li (University of Wisconsin-Madison), and is greatly appreciated. Technical assistance by Christopher Wike is acknowledged with thanks. Thanks as well to Prof. George Horvai (Budapest University of Technology and Economics) for a stimulating discussion of the electrochemical potential of ions in partitioning between immiscible liquids. No funds were received from any outside agency for support of this research. PolyHYDROX-YETHYL A and PolyWAX LP are trademarks of PolyLC Inc.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.chroma.2018.01.038>.

References

- [1] A.J. Alpert, *J. Chromatogr.* 499 (1990) 177–196.
- [2] P. Hemström, K. Irgum, *J. Sep. Sci.* 29 (2006) 1784–1821.
- [3] R. Mountain, *J. Phys. Chem. C* 117 (2013) 3923–3929.
- [4] S.M. Melnikov, A. Höltzel, A. Seidel-Morgenstern, U. Tallarek, *J. Phys. Chem. C* 117 (2013) 6620–6631.
- [5] A.J. Alpert, *Anal. Chem.* 80 (2008) 62–76.
- [6] G. Greco, S. Grosse, T. Letzel, *J. Chromatogr. A* 235 (2012) 60–67.
- [7] Y. Guo, S. Gaiki, *J. Chromatogr. A* 1074 (2005) 71–80.
- [8] Y. Guo, S. Srinivasan, S. Gaiki, *Chromatographia* 66 (2007) 223–229.
- [9] Y. Guo, S. Gaiki, *J. Chromatogr. A* 1218 (2011) 5920–5938.
- [10] N.P. Dinh, T. Jonsson, K. Irgum, *J. Chromatogr. A* 1217 (2011) 5880–5891.
- [11] A.J. Alpert, *Size-Exclusion high-Performance liquid chromatography of small solutes*, in: C.-S. Wu (Ed.), *Column Handbook for Size Exclusion Chromatography*, Academic Press, San Diego, 1999, pp. 249–266.

- [12] N.P. Dinh, T. Jonsson, K. Irgum, J. Chromatogr. A 1320 (2013) 33–47.
- [13] S. Noga, S. Bocian, B. Buszewski, J. Chromatogr. A 1278 (2013) 89–97.
- [14] F. Hofmeister, Arch. Exp. Pathol. Pharmacol. 24 (1888) 247–260.
- [15] K.D. Collins, M.W. Washabaugh, Q. Rev. Biophys. 18 (1985) 323–422.
- [16] M.W. Washabaugh, K.D. Collins, J. Biol. Chem. 261 (1986) 12477–12485.
- [17] W. Bicker, J.Y. Wu, H. Yeman, K. Albert, W. Lindner, J. Chromatogr. A 1218 (2011) 882–895.
- [18] W. Kamichatani, Y. Inoue, M. Saito, Y. Kobayashi, A. Yamamoto, Anal. Sci. 27 (2011) 885–888.
- [19] J. de Grooth, W. Ogieglo, W.M. de Vos, M. Gironès, K. Nijmeijer, N.E. Benes, Eur. Polymer J. 55 (2014) 57–65.
- [20] A.A. Zavitsas, J. Phys. Chem. B 105 (2001) 7805–7817.
- [21] M.Y. Kiriukhin, K.D. Collins, Biophys. Chem. 99 (2002) 155–168.
- [22] B. Tansel, J. Sager, T. Rector, J. Garland, R.F. Strayer, L. Levine, M. Roberts, M. Hummerick, J. Bauer, Sep. Purif. Technol. 51 (2006) 40–47.
- [23] T. Osakai, A. Ogata, K. Ebina, J. Phys. Chem. B 101 (1997) 8341–8348.
- [24] M.E.A. Ibrahim, C.A. Lucy, Talanta 100 (2012) 313–319.
- [25] P. Jandera, Anal. Chim. Acta 692 (2011) 1–25.
- [26] J. Fiala, Separation of Short Nucleotides in Selected Modes of High-performance Liquid Chromatography [Fig. 13] (Batchelor's Thesis), Masaryk University, Dept. of Chemistry, 2015 (Retrieved from) <https://is.muni.cz/th/409295/prif.b/>.
- [27] Y. Kawachi, T. Ikegami, H. Takubo, Y. Ikegami, M. Miyamoto, N. Tanaka, J. Chromatogr. A 1218 (2011) 5903–5919.
- [28] F. Gritti, A. Hölzel, U. Tallarek, G. Guiochon, J. Chromatogr. A 1376 (2015) 112–125.
- [29] M. Gilar, A. Jaworski, J. Chromatogr. A 1218 (2011) 8890–8896 (cf. Table 1).
- [30] A.J. Alpert, O. Hudecz, K. Mechtler, Anal. Chem. 87 (2015) 4704–4711.
- [31] S.M. Melnikov, A. Hölzel, A. Seidel-Morgenstern, U. Tallarek, J. Phys. Chem. C 120 (2016) 13126–13138.