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Effect of salts on retention in hydrophilic interaction chromatography☆

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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.

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-

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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 kosmotropes 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-Å silica (PolyLC Inc., Columbia, MD, USA; item 104HY0301). For HILIC of peptides a column of the same

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