Journal of Chromatography A, 1218 (2011) 882–895

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Retention and selectivity effects caused by bonding of a polar urea-type ligand to silica: A study on mixed-mode retention mechanisms and the pivotal role of solute–silanol interactions in the hydrophilic interaction chromatography elution mode

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ARTICLE INFO

Article history:
Available online 23 October 2010

Keywords:
Hydrophilic interaction chromatography
Ligand surface density
Mixed-mode chromatography
Retention mechanisms
Selectivity
Silanol interactions

ABSTRACT

The separation properties of five silica packings bonded with 1-[3-(trimethoxysilyl)propyl]urea in the range of 0–3.67 μmol m^{-2} were investigated in the hydrophilic interaction chromatography (HILIC) elution mode. An increase of the ligand surface density promoted retention of non-charged polar compounds and even more so for acids. An opposite trend was observed for bases, while the amphoteric compound tyrosine exhibited a U-shaped response profile. An overall partitioning retention mechanism was incompatible with these observations; rather, the substantial involvement of adsorptive interactions was implicated. Support for the latter was provided by column-specific changes in analyte retention and concomitant selectivity effects due to variations of salt concentration, type of salt, pH value, organic modifier content, and column temperature. Silica was more selective for separating compounds differing in charge state (e.g. tyramine *vs.* 4-hydroxybenzoic acid), while in cases where structural differences of solutes resided in non-charged polar groups (e.g. tyramine *vs.* 5-hydroxydopamine, nucleoside *vs.* nucleobase) more selective separations were obtained on bonded phases. Hierarchical cluster analysis of the home-made urea-type and three commercial amide-type bonded packings evinced considerable differences in separation properties. The present data emphasise that the role of the packing material under HILIC elution conditions is hardly just the polar support for a dynamic coating with a water-enriched layer. Three major retention mechanisms are claimed to be relevant on bare silica and the urea-type bonded packings: (i) HILIC-type partitioning, (ii) HILIC-type weak adsorption such as hydrogen bonding between solutes and ligands or solutes and silanols (potentially influenced by individual degrees of solvation, salt bridging, etc.), (iii) strong electrostatic (ionic) solute–silanol interactions (attractive/repulsive). Even when non-charged polar bonded phases are used, solute–silanol interactions should not be discounted, which makes them a prime parameter to be characterised by HILIC column tests. Multi/mixed-mode type separations seem to be common under HILIC elution conditions, associated with a great deal of selectivity increments. They are accessible and controllable by a careful choice of the type of packing, the mobile phase composition, and the temperature.

1. Introduction

Hydrophilic interaction chromatography (HILIC) is gaining acceptance as a promising liquid chromatographic approach for separating polar analytes. The current application field of HILIC, well documented for example by the present thematic issue, by two other special issues [1,2], as well as by further review articles [3–6], ranges from pharmaceutical drug impurity profiling to studies of the metabolome, glycome, and proteome.

HILIC-type separations are commonly claimed to be mainly based on the differential distribution of hydrophilic solutes between a polar stationary phase and a mobile phase of low polarity. The eluent is usually composed of a water-miscible solvent, typically acetonitrile (ACN), and a minor fraction of water (or, occasionally alcohols [7–9]), the latter being the strong eluting part of the mobile phase. Besides solvent composition, the chromato-

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doi:10.1016/j.chroma.2010.10.073

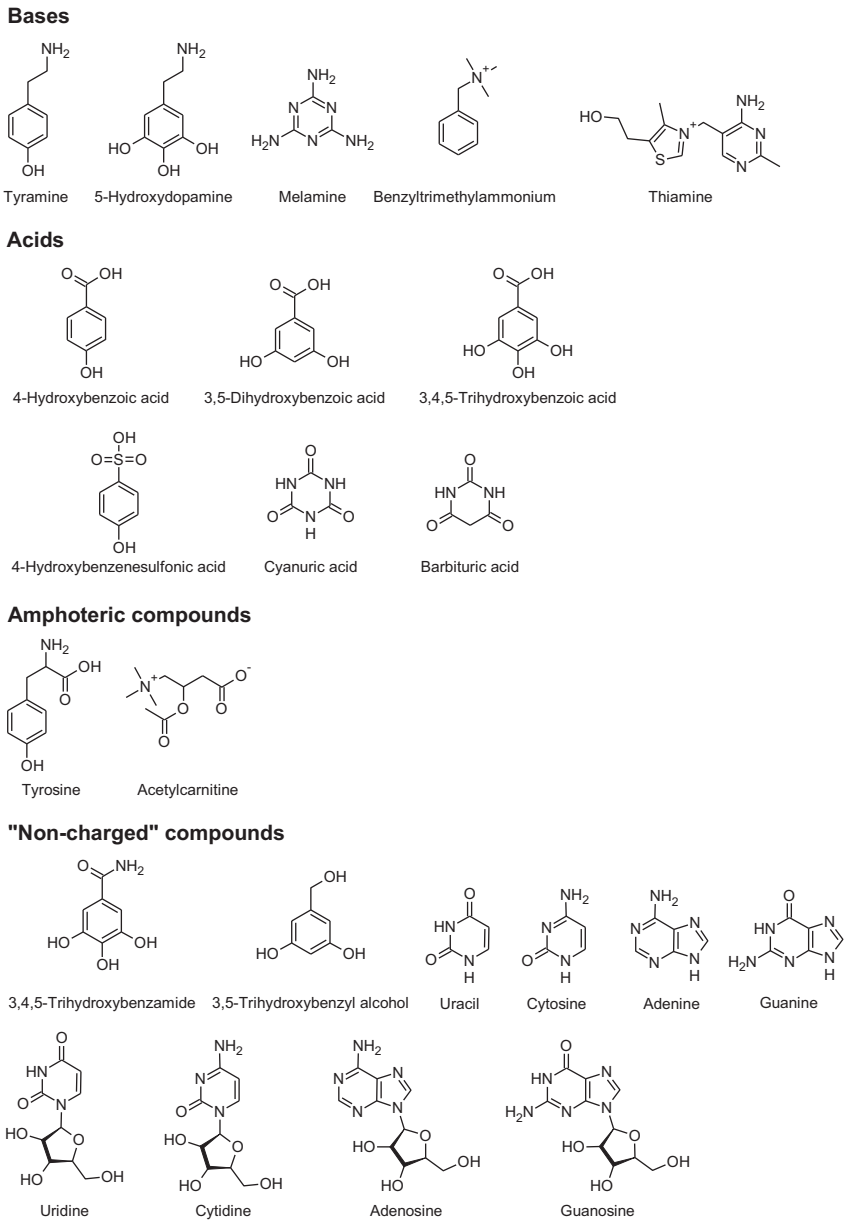

Fig. 1. Structural formulas of the test compounds. "Non-charged" refers to the charge state under the HILIC elution conditions used in most experiments.

graphic retention process may also be affected by the type and amount of salt additives as well as by the pH value and temperature. The large number of different packing materials that can be operated under HILIC elution conditions further extends the toolbox available for method development [3,4,10]. Polarity is the main requirement for a HILIC-type chromatographic material. In the simplest case this is realised by using bare silica. Titania [11,12] and zirconia [13] may be used as well. Silica-based phases bonded with non-charged (e.g. amide, hydroxy, or sulfoxide groups) or cationic/anionic/amphoteric ligands are also suitable materials for HILIC-type separations.

Partitioning and adsorption must be taken into account when describing the mechanisms underlying separations obtained under HILIC-typical conditions. In this context, partitioning refers to the distribution of the analyte between a stagnant layer of water (or alcohol) dynamically enriched on the sorbent surface and the less polar bulk mobile phase. Experimental evidence for the existence of a surface layer rich in water when using eluents with a high fraction of ACN was recently provided for bare silica [14]. Corresponding investigations with bonded packings have not yet been carried out or described. As concerns the chromatographic phase ratio in such a partitioning retention model it is, in the absence of experimental data, debatable to what extent a defined boundary exists between this dynamically adsorbed stationary phase and the mobile phase or if it concerns, to some extent, a gradient of water (in a diffuse layer) having maximal water concentration in proximity to the surface of the sorbent.

In a similar manner to normal-phase chromatography one may consider the development of weak adsorptive interactions such as hydrogen bonding between non-charged functionalities (solute–ligand and others). Attractive or repulsive strong electrostatic (ionic) interactions between charged functionalities of solutes and ligand as well as dissociated silanols may occur as well. As, however, these latter mechanisms are well covered by the terms "ion exchange" and "ion exclusion" and their occurrence is not necessarily linked to HILIC analysis conditions, they should be understood as being distinctly different from "HILIC-type" interactions. Such strong electrostatic interactions can nevertheless

facilitate convenient manipulation of selectivity in the HILIC elution mode as is realised in 'mixed-mode' chromatography [15–19].

Despite this complex mechanistic situation – or indeed because of it – no systematic column characterisation tests have yet been developed for sorbents to be used as HILIC phases. In earlier studies, the authors rationalised separation properties of some commercial and home-made HILIC columns by simple chromatographic tests and chemometric tools [17,20], similar attempts were recently made by others [21,22]. It was demonstrated that ligand structure is an important parameter in determination of retention and selectivity on bonded HILIC materials but it was mentioned as well, as in other studies [7,21,23–30], that in particular adsorptive solute–silanol interactions may also exert a relevant contribution to separation characteristics of polar bonded phases.

In HILIC, the polar ligand and the polar silanol network of the sorbent are both potential sites for the intended (polarity-based) hydrophilic interactions, i.e. (i) a polar backbone for the dynamic enrichment of water as the prerequisite for analyte partitioning and (ii) partners in weak adsorptive interactions with solutes. A variation of the fraction of free silanols and the ligand surface coverage, respectively, may influence the actual HILIC-type retention process and the extent of solute–silanol interactions as well. This particular combination needs to be studied in order to permit the development of a valid HILIC column characterisation test. In the present study this has been done by coating silica with a non-chargeable urea-type ligand in different densities. The packings were characterised chromatographically with different mobile phases and temperatures in a single parameter variation approach using a set of charged and non-charged solutes to elucidate both HILIC-type and non-HILIC-type interactions. Detailed interpretations were possible basically due to the use of (i) bonded packing materials being prepared from the same batch of bare silica and having different but known ligand surface coverage and (ii) some test compounds sharing structural similarities of presumed HILIC interactive sites.

2. Material and methods

2.1. Materials

2.1.1. Chemicals

HPLC grade acetonitrile (ACN), ethanol, methanol, and toluene were obtained from VWR International (Vienna, Austria). HPLC grade water, 1-[3-(trimethoxysilyl)propyl]urea, and 4-dimethylaminopyridine originated from Sigma–Aldrich (Vienna, Austria). Ammonium acetate (NH_4Ac), ammonium formate (NH_4FA), ammonium trifluoroacetate (NH_4F_3Ac), acetic acid, formic acid, trifluoroacetic acid, triethyl amine, and 25% (v/v) aqueous ammonia solution were all of analytical grade and supplied by Sigma–Aldrich. Chromatographic test compounds were each obtained in the highest purity grade available from Sigma–Aldrich (see Fig. 1 for compilation of structural formulas). Daisogel (5 μm, 120 Å, 300 $m^2\,g^{-1}$) was supplied by Daiso Chemical (Osaka, Japan).

2.1.2. Preparation of urea-type column packings

Three grams of bare silica were suspended in 30 mL ethanol. 0.2 or 0.5 or 1.2 mmol 1-[3-(trimethoxysilyl)propyl]urea per gram silica as well as 4-dimethylaminopyridine (0.05 molar fraction with respect to the amount of silane) were added. The mixture was reacted at 70 °C under nitrogen for 19 h. The modified silica materials were each collected by filtration, washed excessively with methanol, and dried at 60 °C for 24 h *in vacuo*. In order to prepare a high-density bonded packing, another 3 g of bare silica were suspended in 30 mL ethanol, 2.5 mmol 1-[3-(trimethoxysilyl)propyl]urea per gram silica were added, and the suspension was evaporated to dryness under reduced pressure. The residue was re-suspended in 30 mL toluene together with 125 μmol 4-dimethylaminopyridine and the mixture was reacted at 130 °C under nitrogen for 19 h. The modified silica material was collected by filtration, washed excessively with methanol, and dried at 60 °C for 24 h *in vacuo*. By determining the nitrogen content of the packings by elemental analysis the surface coverage by the ligand (as sketched in Fig. 2) was calculated. Ligand densities amounted to 0.38, 0.91, 1.81, and 3.67 μmol m^{-2}. Stainless steel columns of the dimension 150 mm × 4 mm ID were slurry-packed with these modified materials and also with bare silica using methanol as slurry and carrier solvent.

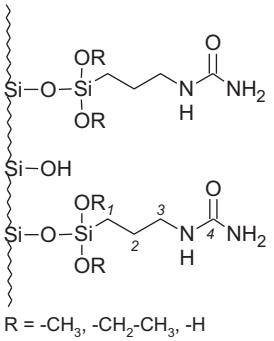

Fig. 2. Structural formula of the chromatographic ligand after being immobilised on silica. Silica surface properties based on solid-state NMR data are shown in Fig. 3.

2.1.3. Commercial amide-type column packings

For chemometric column classification TSKGel Amide-80 (5 μm, 80 Å, 450 $m^2\,g^{-1}$, ligand density not specified; 150 mm × 4.6 mm ID; Tosoh Bioscience, Stuttgart, Germany), XBridge Amide (3.5 μm, 135 Å, 185 $m^2\,g^{-1}$, ligand density 7.5 μmol m^{-2}; 150 mm × 3 mm ID; Waters Corporation, Milford, MA, USA), and Unisol Amide (5 μm, 100 Å, 380 $m^2\,g^{-1}$, ligand density not specified; 150 mm × 4.6 mm ID; Agela Technologies, Newark, DE, USA) were tested together with the home-made packings.

2.1.4. Instrumentation

HPLC runs were performed on a 1200 series HPLC system from Agilent Technologies (Waldbronn, Germany) equipped with a diode array detector. Acetylcarnitine was detected with a corona-charged aerosol detector from ESA Analytical (Aylesbury, UK). Instrument control and data acquisition was performed using Chemstation software from Agilent Technologies. Solid-state ^{29}Si cross polarisation/magic angle spinning (CP/MAS) NMR measurements were performed on a MSL 200 spectrometer from Bruker (Ettlingen, Germany). ^{13}C CP/MAS NMR investigations were carried out on an ASX 300 spectrometer from the same manufacturer.

2.2. Methods

All chromatographic runs were carried out in isocratic elution mode with the flow rate set to 1.00 mL min^{-1}; for the XBridge Amide column a flow rate of 0.5 mL min^{-1} was used. The column compartment was kept at 25 °C except where noted. Test compounds were dissolved in ACN/water (90:10; v/v) at concentration levels ranging from 0.25 to 1 mg mL^{-1}. Sample solutions were injected in 5 μL fractions. Toluene was used as void volume marker. Columns were allowed to equilibrate with about 20 column volumes of each new mobile phase.

Eluents were composed of ACN and aqueous buffers in a volume ratio of 90 to 10 or 80 to 20. The aqueous buffers were prepared by dissolving appropriate amounts of NH_4Ac or NH_4FA or NH_4F_3Ac or triethylamine in water so that the final concentration in the hydro-

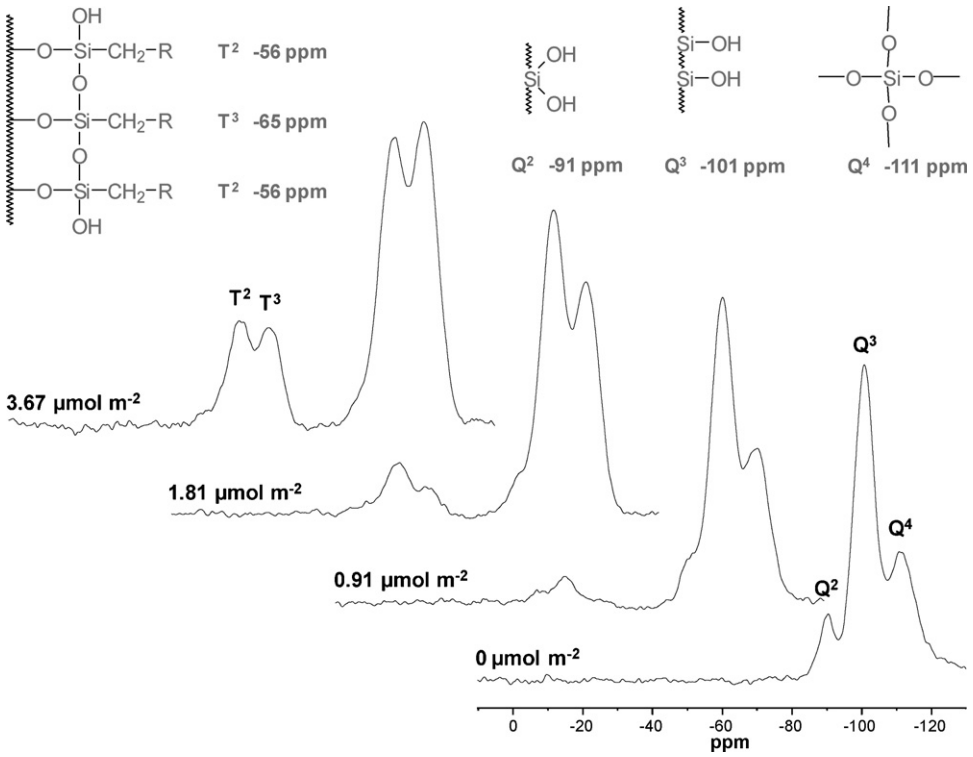

Fig. 3. Solid-state ^{29}Si CP/MAS NMR spectra obtained for bare silica as well as for bonded urea-type packings (0.91, 1.81, 3.67 μmol m^{-2}).

organic mixture amounted to 3.75–30 mM of the desired additive. $^{w}_{w}$pH values, i.e. measured in aqueous buffer prior mixing with ACN, were adjusted accordingly with either acetic acid or formic acid or trifluoroacetic acid or 25% (v/v) aqueous ammonia solution. In the case of triethylamine acetic acid was used to prepare a triethylammonium acetate (TEAAc) buffer *in situ*. For detailed mobile phase compositions the reader is referred to the respective sections in the text as well as to figure legends and table captions.

^{29}Si CP/MAS NMR was executed at sample spinning rates of approximately 3000 Hz. The spectra were recorded using 90° proton pulse lengths of 7.5 μs, contact times of 5 ms, and delay times of 1 s. ^{13}C CP/MAS NMR investigations used sample spinning rates of about 4000 Hz, 90° proton pulse lengths of 3.5 μs, contact times of 3 ms, and delay times of 1 s.

For the purpose of column classification, hierarchical cluster analysis on retention factors k was performed using Number Cruncher Statistical System software version 07.1.13 (NCSS, Kaysville, UT, USA). The group average technique (non-weighted pair group) was applied for data agglomeration and Euclidean distances were used for similarity measurements.

3. Results

3.1. Solid-state NMR spectroscopic characterisation of urea-type packings

Fig. 3 depicts the ^{29}Si CP/MAS NMR spectra of the home-made packings having a ligand surface density of 0, 0.91, 1.81, and 3.67 μmol m^{-2}.

The spectra contained two main groups of resonances. One group represented the peaks of silanediols (Q^2) at −91 ppm, the silanols (Q^3) at −101 ppm, and the siloxanes (Q^4) at −111 ppm. The other group of signals represented the silyl species derived from the ligand bonding reaction, i.e. the trifunctional T groups with partial cross-linking (T^2) at −56 ppm and with complete cross-linking (T^3) at −65 ppm. The fraction of silanediols and silanols diminished, conversely the degree of siloxanes increased when larger amounts of the ligand were bonded to the surface. The extent of silyl cross-linking was estimated by comparison of the relative intensities of the silyl species and it rose with increasing ligand density. This was especially observed with the resonance of the completely cross-linked T^3 group starting to occur at a ligand surface coverage of 1.81 μmol m^{-2} (Fig. 3).

All resonances in ^{13}C CP/MAS NMR spectra appeared in a range between 0 and 180 ppm. Signals of the ligand chain were observed at 9.9 (C1), 24.4 (C2), 44.2 (C3), and 163.2 (C4) ppm (for numbering of C atoms see Fig. 2). A resonance at 17.1 ppm (except the 3.67 μmol m^{-2} bonded packing) gave evidence of an ethoxy moiety. Since the bonding approach up to a ligand surface density of 1.81 μmol m^{-2} involved condensation in ethanol an interchange of methoxy *vs*. ethoxy in the ligand is a plausible explanation for this observation. The increase of cross-linking as was observed by ^{29}Si CP/MAS NMR (*vide supra*) was also reflected in the ^{13}C CP/MAS NMR spectra. The relative signal intensity at 17.1 ppm decreased notably at higher ligand surface coverage, tantamount to a higher degree of cross-linked T^3 groups on the more densely bonded packings.

3.2. Gross retention trends on bare silica and urea-type bonded packings

Retention and selectivity effects caused by changing the ligand density and the residual silanol fraction, respectively, were evaluated by the chromatographic characterisation of bare silica and bonded packings, having 0.38, 0.91, 1.81, and 3.67 μmol m^{-2} of 1-[3-(trimethoxysilyl)propyl]urea attached to the silica surface.

Fig. 4a–c depicts retention curves of solutes with different charge states. Tyramine, a primary aliphatic amine with an aromatic residue, showed a continuous loss of retention when increasing the ligand bonding density. This trend was resembled by the strong base benzyltrimethylammonium cation, albeit changes in retention were more significant. This even caused an inversion of elution order of these two compounds (note the similar y-axis

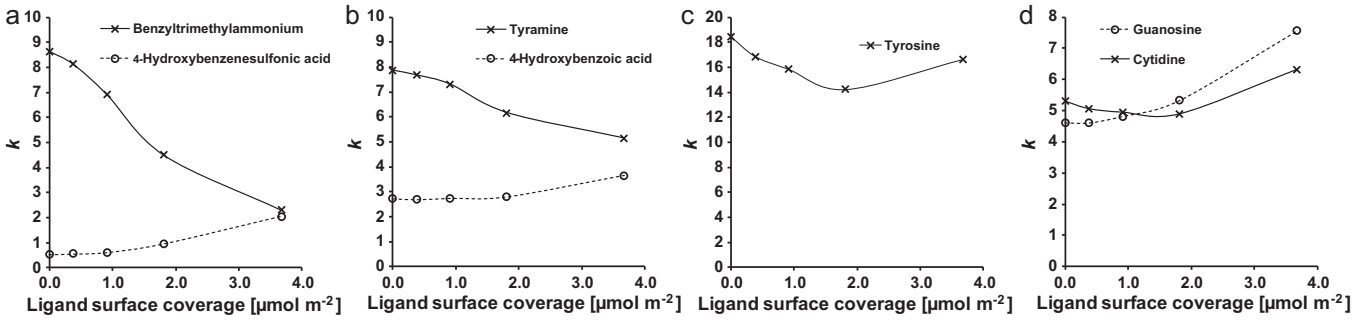

Fig. 4. Change of retention factor k of (a) benzyltrimethylammonium cation (solid line) and 4-hydroxybenzenesulfonic acid (dashed line), (b) tyramine (solid line) and 4-hydroxybenzoic acid (dashed line), (c) tyrosine as well as (d) cytidine (solid line) and guanosine (dashed line) as a function of ligand surface density. Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}$pH = 6.0) (90:10; v/v). Further chromatographic details are given in Section 2.

scaling in Fig. 4a and b). In contrast, 4-hydroxybenzenesulfonic acid and 4-hydroxybenzoic acid were each more strongly retained when the ligand bonding density was increased. This effect became particularly significant at very high ligand surface coverage and it was more pronounced for the sulfonic acid. For tyrosine a U-shaped retention profile was observed while absolute retention varied only by about 23% with the minimum being observed on the 1.81 μmol m^{-2} bonded column (k = 14.2) and the maximum value on bare silica (Fig. 4c).

A change of the elution order of cytidine and guanosine, both assumed to be non-charged under the chosen elution conditions, as a function of ligand surface coverage is shown in Fig. 4d. Cytidine experienced a loss in retention upon introducing the ligand to the silica surface up to a bonding density of 1.81 μmol m^{-2}. On the most densely bonded phase a gain in retention was observed, overall resulting in a slightly U-shaped retention curve. By contrast, retention of guanosine increased steadily.

In an attempt to generalise these findings, the retention of sixteen more solutes, covering non-charged compounds, acids, bases, as well as amphoteric compounds (cf. Fig. 1), was mapped on the bare silica column, an intermediate-dense bonded column (1.81 μmol m^{-2}) and the most densely bonded packing (3.67 μmol m^{-2}). Orthogonal plotting of ln values of retention factors k of all twenty-three test compounds was selected as a simple but instructive output format (Fig. 5).

The results of the most densely bonded packing (3.67 μmol m^{-2}), represented by filled symbols in Fig. 5, and the bare silica column shall be summarised first. For most of the test compounds k values differed by more than 10% on these two phases. All acidic solutes showed stronger retention on the bonded packing (points lying above the dotted 45° equivalence line). A stronger retention on the bonded stationary phase, although usually to a minor degree (7–64%), was also observed for most of the solutes assumed to be non-charged under the chosen elution conditions (mainly nucleobases and nucleosides). In agreement with the trends of tyramine and benzyltrimethylammonium cation discussed above, also other basic solutes were more weakly retained on the bonded phase than on bare silica (represented by points lying below the equivalence line). The strong base thiamine was affected the most (loss in k by 82%). Also the amphoteric compound acetylcarnitine (quaternary amine + carboxylic acid, marked "2" in Fig. 5) was much more weakly retained (−85%) on the bonded phase.

A closer inspection of the data plotted in Fig. 5 revealed that for most solutes k values also changed significantly between the 1.81 and the 3.67 μmol m^{-2} bonded phases. The trends were largely similar to those discussed above, specifically non-charged and acidic solutes were more strongly retained on the more densely bonded packing (illustrated in Fig. 5 by arrow pointing upwards) while bases, especially quaternary amines, were more weakly retained (arrow pointing downwards). Overall, the retention trends observed with the initial small compound set (Fig. 4) were confirmed by the larger compound set (Fig. 5).

As concerns chromatographic selectivity α, a profound role of ligand surface coverage was evident as well. Table 1 lists selectivity values of structurally related solutes as obtained on bare silica as well as on the 1.81 and the 3.67 μmol m^{-2} bonded packings. Differences in the charge state between analytes led to higher selectivities on bare silica (e.g. tyramine *vs.* 4-hydroxybenzoic acid, 5-hydroxydopamine *vs.* 3,4,5-trihydroxybenzamide), while bonded phases were favourable for the separation of solutes differing in non-charged polar groups (e.g. tyramine *vs.* 5-hydroxydopamine, nucleoside *vs.* nucleobase). A gain in selectivity due to a change in the ligand surface density was not always associated with a gain in absolute retention and *vice versa*. For example, k values of 5-hydroxydopamine and tyramine decreased on more densely bonded phases while selectivity increased from

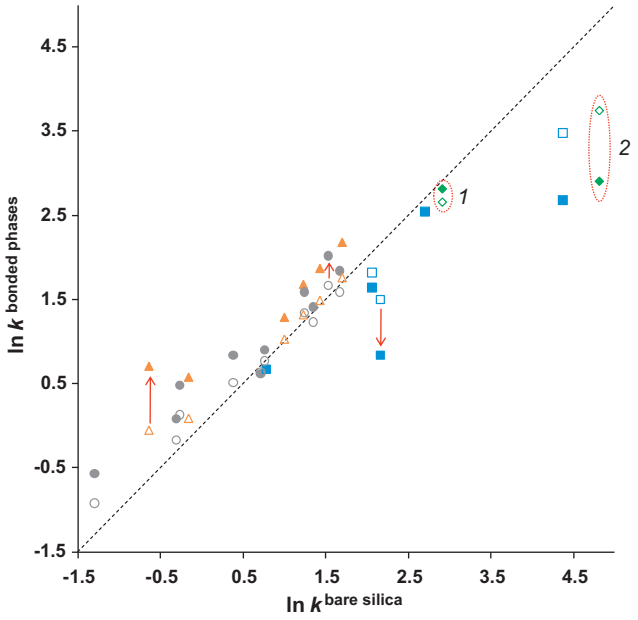

Fig. 5. Orthogonality plots of retention factors (ln k *vs.* ln k) of non-charged (◯, grey), acidic (△, orange), basic (□, blue), and amphoteric (◊, green; *1*. . .tyrosine, *2*. . .acetylcarnitine) analytes observed on bonded packings (open symbols represent retention on 1.81 μmol m^{-2} bonded column, filled symbols represent retention on 3.67 μmol m^{-2} bonded column) *vs.* bare silica. Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}$pH = 6.0) (90:10; v/v). Further chromatographic details are given in Section 2. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 1
Selectivity values α obtained on packings of different ligand surface density. Mobile phase: ACN/(100 mM aqueous NH_4Ac, ${}^{w}_{w}pH = 6.0$) (90:10; v/v). Further chromatographic details are given in Section 2.

Compound pair	Chromatographic selectivity Ligand surface density [$\mu mol\,m^{-2}$]		
	0	1.81	3.67
Tyramine/4-hydroxybenzoic acid	2.89	2.21	1.41
Tyrosine/4-hydroxybenzoic acid	6.77	5.09	4.57
4-Hydroxybenzoic acid/4-hydroxybenzenesulfonic acid	5.14	2.93	1.78
5-Hydroxydopamine/3,4,5-trihydroxybenzamide	19.46	11.16	7.81
5-Hydroxydopamine/tyramine	1.89	2.07	2.46
3,4,5-Trihydroxybenzoic acid/4-hydroxybenzoic acid	2.02	2.08	2.43
3,5-Dihydroxybenzoic acid/4-hydroxybenzoic acid	1.26	1.35	1.48
Guanosine/guanine	1.33	1.40	1.55
Adenosine/adenine	1.05	1.15	1.33

1.89 on bare silica to 2.46 on the 3.67 $\mu mol\,m^{-2}$ bonded packing. The opposite trend was observed for 4-hydroxybenzoic acid *vs*. 4-hydroxybenzenesulfonic acid.

To investigate the role of ligand surface coverage more comprehensively, especially from a mechanistic point of view, additional studies were carried out at some different elution conditions.

3.3. Effects of elution parameters in context of ligand surface coverage

3.3.1. Variation of salt concentration at fixed ${}^{w}_{w}pH$ value

Variations of the salt concentration in the mobile phase caused selectivity differences as exemplified in Table 2 with eluents containing either 10 or 20 mM NH_4FA (90% ACN, ${}^{w}_{w}pH = 7.0$).

A decrease of selectivity was observed for tyramine *vs*. 4 hydroxybenzoic acid on all packings when using higher salt concentrations, while selectivity of 3,4,5-trihydroxybenzoic acid *vs*. 4-hydroxybenzoic acid increased. The extent of these effects depended on ligand surface coverage. As will be discussed in Section 4 changes in the salt concentration may impact hydrophilic and ionic interactions. In case of charged compounds shielding of acidic surface silanols seems to be particularly relevant, considering both attractive (basic solute–silanol) and repulsive (acidic solute–silanol) interactions.

A more detailed study on the effects of salt concentration was carried out using NH_4Ac instead of NH_4FA and a salt content of 3.75–30 mM (90% ACN, ${}^{w}_{w}pH = 6.0$). Effects on retention are summarised in the following (Fig. 6).

The retention of benzyltrimethylammonium cation decreased steadily upon increasing the concentration of NH_4Ac, especially on packings of low ligand density (Fig. 6a). A similar trend was observed for tyramine on bare silica up to 15 mM NH_4Ac while the eluent containing 30 mM NH_4Ac already led to a virtually identical value of k compared to the mobile phase with 15 mM NH_4Ac (Fig. 6b). On the 3.67 $\mu mol\,m^{-2}$ bonded column the eluent containing 30 mM NH_4Ac even led to a more strongly retention than the 3.5 mM NH_4Ac eluent, tantamount to a pronounced 'U-shaped' retention curve (Fig. 6b).

4-Hydroxybenzenesulfonic acid, cytidine, and guanosine were each stronger retained at higher salt concentrations. However, a concentration of 15 mM NH_4Ac still delivered a lower retention of 4-hydroxybenzenesulfonic acid on bare silica ($k = 0.70$) compared to the use of 3.75 mM NH_4Ac on the 3.67 $\mu mol\,m^{-2}$ bonded packing ($k = 1.08$) (Fig. 6c). While for 4-hydroxybenzenesulfonic acid pronounced effects on retention were already observed at lower salt concentrations, the retention of non-charged nucleosides was only affected significantly when the salt concentration exceeded 15 mM NH_4Ac (Fig. 6d).

3.3.2. Variation of the type of salt at fixed ${}^{w}_{w}pH$ value

Aqueous solutions of 100 mM NH_4Ac, NH_4FA, NH_4F_3Ac, or TEAAc, all being adjusted to ${}^{w}_{w}pH = 5.0$ were mixed with ACN in a 10–90 volume ratio. This resulted in different ${}^{s}_{w}pH$ values, namely 7.7 (NH_4Ac), 7.6 (TEAAc), 7.2 (NH_4FA), 5.1 (NH_4F_3Ac). The chromatographic differences observed with these four different HILIC eluents should therefore not be interpreted as a sole "type of salt" effect but they should better be considered to be *integrated* ${}^{s}_{w}pH$/salt-effects.

4-Hydroxybenzenesulfonic acid showed strongest retention with the eluent containing NH_4F_3Ac while weakest retention was found with TEAAc (Fig. 7a). This general trend was not affected by the ligand bonding density. On the 3.67 $\mu mol\,m^{-2}$ packing retention of this strong acidic analyte adopted a value of $k = 6.5$, while it remained between 1.0 and 2.5 for the other salts. Opposed to that, benzyltrimethylammonium cation showed strongest retention with NH_4Ac (except of the 3.67 $\mu mol\,m^{-2}$ packing where a slightly stronger retention was obtained with the TEAAc eluent), while lowest values on all packings were delivered by the mobile phase containing NH_4F_3Ac (Fig. 7b). For weak acids and bases, more diverse effects due to changes in the type of salt were observed. For instance, retention of 4-hydroxybenzoic acid was minimal using NH_4F_3Ac as eluent additive and maximal with NH_4Ac (Fig. 7c). This finding is in sharp contrast to what was found for its strongly acidic analogue 4-hydroxybenzenesulfonic acid (*vide supra*). Notably, the use of TEAAc caused 4-hydroxybenzoic acid to elute earlier on bonded packings compared to bare silica, while for NH_4FA and NH_4F_3Ac the trend resembled that with NH_4Ac. The situation was the inverse for the weak base tyramine (Fig. 7d) because the loss in retention on more densely bonded packings observed with NH_4Ac was also found with NH_4FA and NH_4F_3Ac but not for TEAAc. In a similar manner to 4-hydroxybenzoic acid, lowest absolute retention values of tyramine were obtained when using NH_4F_3Ac as additive.

The retention of non-charged solutes (studied with guanosine, cytidine, guanine, and 3,4,5-trihydroxybenzamide) was less affected by the type of salt. Values of k increased in the order $NH_4F_3Ac < TEAAc < NH_4FA < NH_4Ac$ on all packings (Fig. 7e and f). The gain in retention upon exchanging NH_4F_3Ac by NH_4Ac was highest on bare silica (e.g. gain in k by 50% for guanine and by about 100% for cytidine, guanosine, and 3,4,5-trihydroxybenzamide). Overall, the range of k spanned by using the different salt additives was much higher for the charged solutes discussed above (up to 10-fold) compared to the non-charged test compounds.

3.3.3. Variation of ${}^{w}_{w}pH$ at fixed salt concentration

Selectivity values obtained at ${}^{w}_{w}pH = 4.0$ and 7.0 using 10 mM NH_4FA as salt additive are given in Table 2. It is noted that ${}^{w}_{w}pH = 7.0$ is outside the buffer range of NH_4FA, although it was still possible to obtain quite reproducible k values. The separation of chargeable solute pairs was more affected by ${}^{w}_{w}pH$ variations than that of non-charged solute pairs as well as charged compound pairs differing only in non-charged functionalities. For example, at ${}^{w}_{w}pH = 7.0$ selectivity of tyramine *vs*. 4-hydroxybenzoic acid was 2.97 on bare silica while at ${}^{w}_{w}pH = 4.0$ this figure rose to 7.52. On the 3.67 $\mu mol\,m^{-2}$ packing selectivity increased from 1.85 to 3.77. Particularly profound effects were observed in separations involving amphoteric tyrosine (Table 2). Nucleoside/nucleobase separations were virtually unaffected by ${}^{w}_{w}pH$ variations, especially when using densely bonded phases.

Working at a ${}^{w}_{w}pH$ value of 3.0 with different salts showed for example that, in comparison to ${}^{w}_{w}pH = 5.0$ (Section 3.3.2), the

Table 2
Selectivity values α obtained on packings of different ligand surface density and under different elution conditions (single parameter variation approach). Further chromatographic details are given in Section 2.

Compound pair	Elution condition	Chromatographic selectivity Ligand surface density [μmol m^{-2}]		
		0	1.81	3.67
Tyramine/4-hydroxybenzoic acid	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	2.97	2.42	1.85
	80% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	2.18	1.49	1.14
	90% ACN, 20 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	1.48	1.31	1.16
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 4.0, 25 °C	7.52	5.12	3.77
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 50 °C	5.35	3.84	2.83
Tyrosine/4-hydroxybenzoic acid	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	9.77	7.67	7.67
	80% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	3.33	2.70	2.59
	90% ACN, 20 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	14.99	13.58	12.25
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 4.0, 25 °C	22.05	16.57	16.26
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 50 °C	15.08	10.92	10.56
3,4,5-Trihydroxybenzoic acid/4-hydroxybenzoic acid	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	2.18	2.44	3.04
	80% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	1.49	1.70	1.89
	90% ACN, 20 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	5.62	5.49	5.64
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 4.0, 25 °C	2.25	2.81	3.33
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 50 °C	2.02	2.55	3.07
Guanosine/guanine	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	1.25	1.39	1.56
	80% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	1.10	1.20	1.31
	90% ACN, 20 mM NH_4FA, $^{w}_{w}$pH = 7.0, 25 °C	2.27	2.22	2.19
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 4.0, 25 °C	1.21	1.38	1.55
	90% ACN, 10 mM NH_4FA, $^{w}_{w}$pH = 7.0, 50 °C	1.11	1.28	1.47

retention of 4-hydroxybenzoic acid was more affected when using NH_4FA (k values by 78–88% higher at $^{w}_{w}$pH 5.0) instead of NH_4F_3Ac (variation in k less than 25%), albeit absolute retention was already much lower in the case of NH_4F_3Ac. Tyramine was about 20% more strongly retained at $^{w}_{w}$pH = 5.0 compared to $^{w}_{w}$pH = 3.0 with NH_4FA while with NH_4F_3Ac stronger retention between 8% on bare silica and 40% on the most densely bonded packing was obtained at $^{w}_{w}$pH = 3.0.

3.3.4. Variation of modifier content

Most analytes experienced a loss in retention between 50 and 70% in terms of k when increasing the amount of water in the eluent from 10 to 20% (v/v) (studied with overall 10 mM NH_4FA, $^{w}_{w}$pH = 7.0). The most pronounced decrease in k (loss up to 85%) was observed for amphoteric tyrosine. Retention decreased in direction about 5% more on the bare silica column compared to the 3.67 μmol m^{-2} bonded packing. Selectivity values were generally negatively influenced by an increase of the water fraction (Table 2). There were no other appreciable trends, either related to specific structural elements of analytes or the impact of ligand surface coverage. More systematic experiments were therefore not carried out, not least because of the low absolute retention observed for most analytes already at 20% (v/v) water in the eluent which made an exact determination of k at higher fractions of water difficult.

3.3.5. Variation of temperature

Temperature effects were studied in the range of 15–55 °C column compartment temperature. Retention of non-charged compounds and acids decreased at higher temperatures, tantamount to negative retention enthalpies ΔH (Table 3).

Straight lines in van't Hoff plots were not obtained for all compounds (reflected by lower correlation coefficients), especially considering the behaviour of bases on densely bonded packings, and in such cases ΔH and ΔS values are only rough approximations for the thermodynamic properties of the separation process. It has to be considered as well that variations in temperature may also have an impact on the phase ratio which may be a particularly critical factor under HILIC elution conditions [7]. ΔH values of guanosine, cytidine, and guanine were to a lesser extent affected by the ligand surface coverage (e.g. $\Delta H^{\text{guanosine}}$ (bare silica) = −13.9 kJ mol^{-1}, $\Delta H^{\text{guanosine}}$ (3.67 μmol m^{-2} bonded column) = −12.7 kJ mol^{-1}), while for 4-hydroxybenzenesulfonic acid a pronounced change from −40.3 kJ mol^{-1} on bare silica to −6.9 kJ mol^{-1} on the 3.67 μmol m^{-2} packing was observed. The weak base tyramine showed a slightly negative retention enthalpy on the 3.67 μmol m^{-2} phase, which turned to a positive value at lower ligand densities and became maximal on the bare silica column (ΔH = 6.0 kJ mol^{-1}). This column-specific temperature-dependent retention trend of tyramine is depicted in Fig. 8. Benzyltrimethylammonium cation exhibited positive values

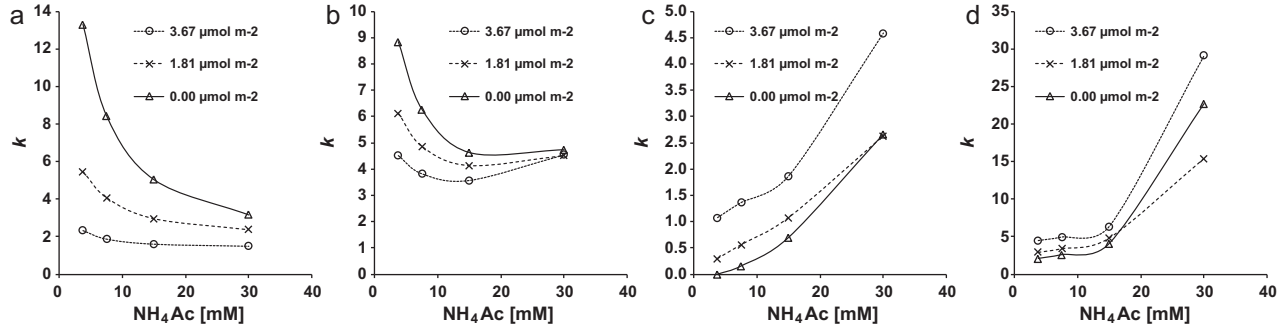

Fig. 6. Change of retention factor k of (a) benzyltrimethylammonium cation, (b) tyramine, (c) 4-hydroxybenzenesulfonic acid, and (d) guanosine on bare silica (solid line), the 1.81 μmol m^{-2} bonded packing (dashed line), and the 3.67 μmol m^{-2} bonded packing (fine dashed line) as a function of the amount of NH_4Ac in the eluent. Mobile phase: ACN/(3.75–300 mM aqueous NH_4Ac, each $^{w}_{w}$pH = 6.0) (90:10; v/v). Further chromatographic details are given in Section 2.

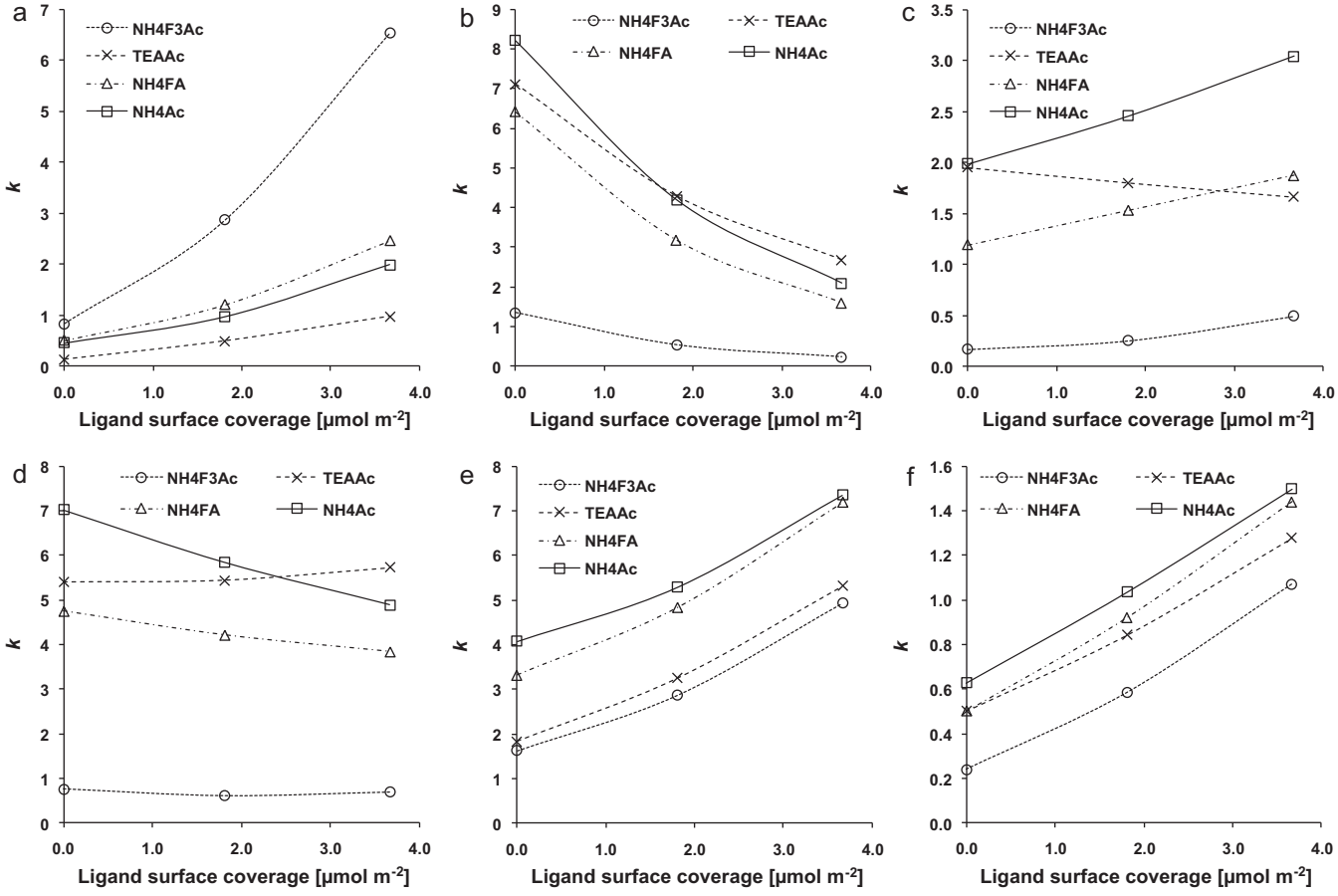

Fig. 7. Change of retention factor k of (a) 4-hydroxybenzenesulfonic acid, (b) benzyltrimethylammonium cation, (c) 4-hydroxybenzoic acid, (d) tyramine, (e) guanosine, and (f) 3,4,5-trihydroxybenzamide as a function of ligand surface density and type of salt in the eluent, *viz.* NH_4Ac (solid line), NH_4FA (dash-dotted line), NH_4F_3Ac (fine dashed line), TEAAc (dashed line). Mobile phase: ACN/(100 mM salt in aqueous solution, each $^{w}_{w}$pH = 5.0) (90:10; v/v). Further chromatographic details are given in Section 2.

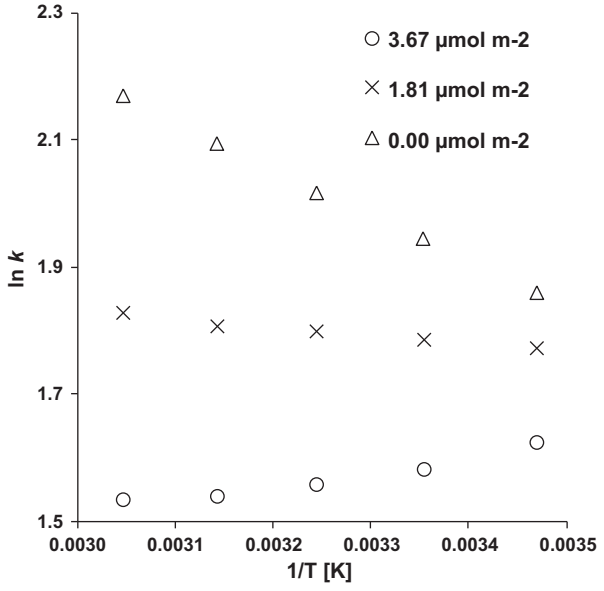

Fig. 8. Van't Hoff plots $\ln k$ *vs.* $1/T$ [K] of tyramine as observed on three different packings, i.e. bare silica (△), 1.81 µmol m^{-2} bonded column (x), 3.67 µmol m^{-2} bonded column (○). Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}$pH = 5.0) (90:10; v/v). Temperature range, 15–55 °C. Further chromatographic details are given in Section 2.

of ΔH on all phases, being higher at lower ligand surface coverage as with tyramine (Table 3).

To illustrate the practical implication of temperature effects, both with respect to the type of analyte and the ligand surface coverage, some $\ln \alpha$ *vs.* $1/T$ plots are provided by Fig. 9.

For example, higher temperature as well as lower ligand densities exerted negative effects on α of guanosine/guanine separation on all phases (Fig. 9a and Table 2). An opposite

Table 3
Retention enthalpies ΔH [kJ mol^{-1}], retention entropies ΔS [J mol^{-1} T^{-1}], and correlation coefficients r^2 as obtained from van't Hoff plots in the temperature range 15–55 °C on packings of different ligand surface density. Values are means of three independent experimental series; %RSD values were 0.31–2.73 for ΔH and 0.33–2.10 for ΔS. Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}$pH = 5.0) (90:10; v/v). Further chromatographic details are given in Section 2.

Analyte		Ligand surface density [µmol m^{-2}]		
		0	1.81	3.67
Guanosine	ΔH	−13.9	−14.0	−12.7
	ΔS	−34.8	−32.9	−26.0
	r^2	0.989	0.986	0.986
4-Hydroxybenzenesulfonic acid	ΔH	−40.3	−15.4	−6.9
	ΔS	−142.0	−51.5	−17.2
	r^2	0.975	0.994	0.974
Benzyltrimethylammonium cation	ΔH	11.4	5.5	1.1
	ΔS	55.8	30.7	10.1
	r^2	0.992	0.991	0.590
Tyramine	ΔH	6.0	1.0	−1.8
	ΔS	36.4	18.3	7.3
	r^2	0.999	0.978	0.922

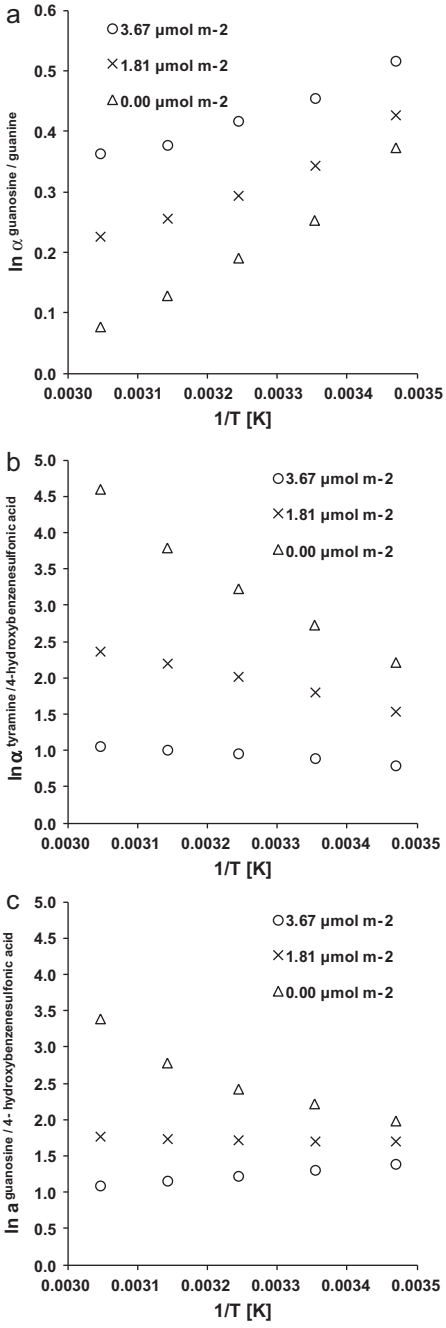

Fig. 9. Plots of ln α *vs.* $1/T$ [K] of (a) guanosine/guanine, (b) tyramine/4-hydroxybenzenesulfonic acid, and (c) guanosine/4-hydroxybenzenesulfonic acid as observed on three different packings, i.e. bare silica (△), 1.81 μmol m^{-2} bonded column (x), 3.67 μmol m^{-2} bonded column (○). Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}$pH = 5.0) (90:10; v/v). Temperature range, 15–55 °C. Further chromatographic details are given in Section 2.

temperature effect was observed for the separation of tyramine and 4-hydroxybenzenesulfonic acid, two solutes of little similarity with respect to the charge state compared to the compound pair guanosine and guanine (Fig. 9b). The value of $\alpha^{\text{tyramine/4-hydroxybenzenesulfonic acid}}$ was close to 100 at 55 °C and when using the bare silica phase while it was minimal on the 3.67 μmol m^{-2} packing (α = 2.20–2.86). The selectivity of guanosine and 4-hydroxybenzenesulfonic acid showed a similar trend. However, while on bare silica selectivity was significantly improved as well by temperature ($\alpha^{15\,°C}$ = 7.26, $\alpha^{55\,°C}$ = 29.4), this effect strongly diminished on the 1.81 μmol m^{-2} bonded column, and it was even inverted (i.e. loss in α at higher temperature) on the 3.67 μmol m^{-2} packing ($\alpha^{15\,°C}$ = 3.99, $\alpha^{55\,°C}$ = 2.98) (Fig. 9c). Thus, in a similar manner to mobile phase parameters it became obvious that also a variation of the temperature (albeit studied herein only in a relatively narrow range) has the potential to influence HILIC separations considerably, again in an analyte- and column-specific way.

3.4. Hierarchical cluster analysis of home-made and commercial HILIC packings

In order to bridge separation results obtained on bare silica and the home-made urea-type columns with those of some commercial packings, chemometric similarity analysis was carried out. Three amide-type phases were selected for this purpose, namely TSKGel Amide-80, Unisol Amide, and XBridge Amide. It is noted that apart from a lack of information on the ligand structures and bonding strategies of the commercial materials these packings also differed (according to the manufacturers) considerably in specific surface area (185–450 m^2 g^{-1}; base silica of home-made packings: 300 m^2 g^{-1}). Differences in separation properties may thus not necessarily be caused only by the surface functionalisation. Fig. 10 depicts the plot obtained by hierarchical cluster analysis on *k* values of eight test solutes, a set that (except for guanine) was used earlier by the authors to classify polar columns under gradient HILIC elution conditions [20].

Bare silica and the 0.38 μmol m^{-2} bonded phase showed the greatest similarity and formed a sub-cluster. Together with the 0.91 and the 1.81 μmol m^{-2} bonded packings these four columns were clustered together but they were still separated from the 3.67 μmol m^{-2} column and the three commercial bonded packings. The 3.67 μmol m^{-2} bonded phase was most similar to the Unisol Amide phase. The separation properties of TSKGel Amide-80 were markedly different from these two columns and also from the XBridge Amide packing. This is in agreement with earlier studies, in which the chromatographic properties of this packing were reported to be rather dissimilar to various other polar bonded phases [17,19,21,29,30]. For example, absolute retention of guanine, guanosine, and cytidine was much higher on TSKGel Amide-80 compared to all other packings. The retention of tyramine on TSKGel Amide-80 was close to the value obtained on the 1.81 μmol m^{-2} bonded urea-type column while 4-hydroxybenzoic acid was retained more strongly by almost a factor of two. Accordingly, selectivity differences between the columns were manifested for most solute pairs. This confirmed the prime importance of column selection during HILIC method development in addition to a dedicated optimisation of elution conditions.

4. Discussion

4.1. General considerations on separation properties of bare silica and urea-type bonded packings

Manufacturers of commercial HILIC columns usually provide only minimal information on phase chemistry, ligand surface density, end-capping strategies, etc. This gap of knowledge, together with the issue of having different silica base materials, generates considerable uncertainty in mechanistic interpretations of chromatographic data obtained on commercial columns. To counter this problem in the framework of the present study, it was decided to prepare silica packings bonded with a controlled amount of a polar ligand using the same base silica material. Type B Daisogel silica was functionalised with 1-[3-(trimethoxysilyl)propyl]urea. The motivation of introducing this urea-type ligand was driven by the following considerations: (i) the ligand is non-chargeable under typical HILIC elution conditions and this was preferred over using a

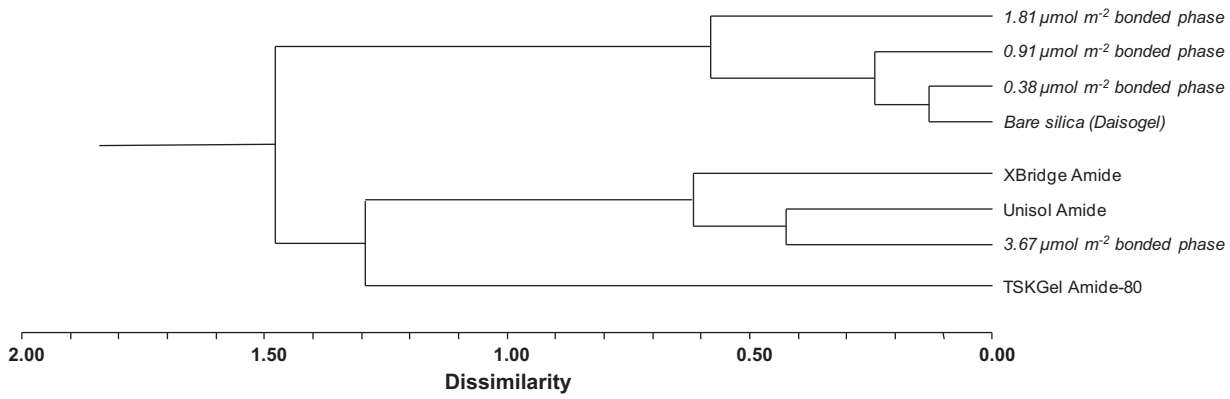

Fig. 10. Classification of home-made urea-type and selected commercial amide-type HILIC packings by hierarchical cluster analysis. Retention factors *k* of tyramine, benzyltrimethylammonium cation, 4-hydroxybenzoic acid, 4-hydroxybenzenesulfonic acid, tyrosine, guanine, cytidine, and guanosine were used as input parameters. Mobile phase: ACN/(100 mM aqueous NH_4Ac, $^{w}_{w}pH = 6.0$) (90:10; v/v). Further chromatographic details are given in Section 2.

chargeable one in order to avoid ionic solute–ligand interactions that otherwise could mimic or shield electrostatic forces stemming from dissociated surface silanols, (ii) it is well known that the highly polar urea strongly adsorbs water due to its superb hydrogen bonding properties resulting in a high degree of solvation, (iii) there was no requirement for additional chemical reactions after ligand immobilisation (such as for example needed in the case of preparation of diol ligands from epoxy-type bonded packings), (iv) a convenient determination of ligand surface coverage via nitrogen analysis was possible. Solid-state ^{29}Si CP/MAS NMR data confirmed that bonding of the polar ligand to silica had distinct effects on surface silicon species (Section 3.1; Fig. 3). Higher ligand density was thus not only tantamount to a change in the total amount of Si–OH functionalities but the accessibility and acidity of residual silanols was likely affected as well.

A water-rich layer can be formed dynamically on bare silica under HILIC elution conditions as has been shown by McCalley and Neue [14]. Owing to the fact that the retention of sugars on an amide-bonded packing was found to decrease with increasing pressure, which was explained by a gain in solute hydration when entering the stationary phase [31], one may speculate that such water-rich layers are present as well on bonded packings. Attaching the polar urea-type ligand to the silica surface in different densities may in that case alter the microstructure and thickness of this water-rich layer because the water binding capabilities of the remaining surface silanols and urea-functionalities are likely somewhat different. Moreover, a larger degree of ligand present on the surface may increase the probability of polarity-based solute–ligand interactions (basically encompassing hydrogen bonding and dipole–dipole forces) which, again due to structural differences between surface silanols and the urea-type ligand, will be of a different nature than weak solute–silanol interactions (not to be confused with non-HILIC-type strong ionic solute–silanol interactions). It is debatable whether these weak adsorptive interactions occur directly or whether indirect interactions between solvation shells of ligands/silanols and solutes appear as well. The latter are regarded to be a precursor for what is called "partitioning" and this emphasises that a clear distinction between partitioning-based and weak adsorptive interactions under HILIC elution conditions is difficult or even impossible [10].

A relevant role of weak adsorptive solute–ligand interactions could help explain the inversion of elution order of (supposed) non-charged cytidine and guanosine at intermediate ligand densities as depicted in Fig. 4d. However, the same may result in case cytidine behaves as a very weak base for which ionic solute–silanol interactions are of additional relevance (*vide infra*), while guanosine does not (using calculated p*K* values of solutes and pH values of mobile phases to clarify this situation seems not fully appropriate due to the unknown pK shifts occurring in the ACN-rich eluent). Overall, the experimental data were not conclusive on the relative contributions of partitioning and weak adsorption and targeted investigations on this specific problem were beyond the scope of the present study. In agreement with a number of previous studies, for example ref. [3,7,10,17,20,25,26,28,32], it is therefore postulated that "HILIC-type" interactions, as will be referred to in the following, are combinations of partitioning and weak adsorption (such as hydrogen bonding) and these should be understood to be different from non-HILIC-type strong electrostatic (ionic) interactions.

4.2. Mechanistic interpretations of chromatographic data of bare silica and urea-type bonded packings

4.2.1. Retention principle of non-charged compounds

A gain in retention of non-charged solutes was observed when using packings of a higher ligand density. In a pure partitioning-based retention model this finding is explained by an increased volume of the water-rich layer induced by the ligand bonding and being tantamount to a change in chromatographic phase ratio. In case adsorption is additionally taken into account, higher retention can be ascribed to the occurrence of solute–ligand interactions. These may be stronger than solute–silanol interactions because of intrinsic structural differences and/or, as would be the case for bare silica, the solute does not need to penetrate the whole water-rich layer until it reaches adsorptive sites on the silica surface.

The extent of retention of non-charged compounds and the elution order of charged compounds differing only in non-charged polar functionalities was related to compound polarity. For example, with the eluent ACN/(100 mM aqueous NH_4FA, $^{w}_{w}pH = 4.0$) (90:10; v/v) the nucleoside guanosine (log $D^{pH\ 4.0} = -1.72$; data taken from SciFinder scholar database whereby all values were calculated at 25 °C with ACD/Labs Software V8.14, Advanced Chemistry Development Inc., Toronto, Canada) was more strongly retained than its corresponding nucleobase guanine (log $D^{pH\ 4.0} = -1.09$) and this trend was similar to other nucleoside/nucleobase-pairs. Also the elution order of 4-hydroxybenzoic acid (log $D^{pH\ 4.0} = 1.32$) < 3,5-dihydroxybenzoic acid (log $D^{pH\ 4.0} = 0.80$) < 3,4,5-trihydroxybenzoic acid (log $D^{pH\ 4.0} = 0.74$) followed the expected trend. It is, however, emphasised that log D/P values can only be regarded as a simplistic concept for estimating the relative strength of HILIC-type interactions because the underlying molecular processes of HILIC-type retention and their correlations with compound polarity are not sufficiently known yet. Severe limitations became obvious in the

case of charged solutes, where other types of interaction come into effect (*vide infra*). Pertinent work of Jinno and co-workers demonstrated, however, that when using more subtle molecular descriptors a prediction of retention under HILIC elution conditions still seems to be possible to some extent [8,24,33–37].

It is known that salt can promote the retention of non-charged compounds under HILIC elution conditions, see for example Refs. [7,29]. This was revealed by the present study in the range of 3.75–30 mM NH_4Ac. An additive effect with ligand density was obvious as well (Fig. 6). HILIC-type partitioning may be influenced by higher salt concentrations because in such case more solvated salt ions enter the water-rich layer, tantamount to a gain in layer thickness. Due to the increased stationary phase volume and maybe additionally facilitated by salt bridging [38], analyte partitioning into this layer is then supported. As concerns adsorptive retention increments, such a gain in the dwell time of the analytes staying in the water-rich layer may directly favour the development/strengthening of solute–ligand (and maybe also solute-salt–ligand or solute-(salt)–silanol) interactions, simply because of an increased probability that solutes come in close proximity to ligand/silanol functionalities. Macroscopically, all these processes lead to higher retention. An impact on chromatography may also come from an increased degree of ion-pairing of acidic silanols with NH_4^+ when using higher concentrations of NH_4Ac, which will alter surface polarity.

The use of NH_4F_3Ac in the mobile phase weakened absolute retention of non-charged solutes compared to NH_4Ac, NH_4FA, and TEAAc. This finding may be explained by two peculiarities of NH_4F_3Ac buffers: (i) NH_4F_3Ac delivers much lower ${}^{s}_{w}$pH (and accordingly ${}^{s}_{s}$pH) values when using buffers being standardised by ${}^{w}_{w}$pH (studied at ${}^{w}_{w}\text{pH} = 3.0$ and 5.0) causing a larger degree of silanol protonation (Section 3.3.2) [39]. This reduces surface polarity, impairs water–silanol interactions, and ultimately leads to a loss in volume of the water-rich layer. As has been demonstrated (Fig. 7), this effect is alleviated when using packings with a higher ligand surface density because then HILIC-type retention does not only rely on interactions of water (prerequisite for partitioning) or the analytes with the silica surface. (ii) A water-rich layer containing trifluoroacetate is supposed to be more lipophilic than layers containing acetate or formate. Consequently, polarity differences between mobile phase and stagnant water-rich layer diminish when using NH_4F_3Ac as salt additive.

4.2.2. Retention principle of basic compounds

The retention process of basic solutes is outlined for the strongly basic benzyltrimethylammonium cation and the weak base tyramine. It appeared to be composed of HILIC-type interactions and attractive strong electrostatic interactions between cationic solutes and dissociated surface silanols.

The ionic interaction increment was assessed according to the concept recently proposed by McCalley [28] as follows. At infinite counter-ion concentration ion exchange processes are postulated to be completely suppressed. In case no other retention processes would be at work for basic solutes under these hypothetical conditions a plot of *k vs.* the inverse counter-ion concentration results in a straight line passing through the origin (see Ref. [28] for theoretical background), while a positive intercept of this plot is tantamount to *k* values > 0 at infinite counter-ion concentration, being equivalent to the presence of other (non-ionic) retention mechanisms.

This intuitive concept was applied to retention data of tyramine and benzyltrimethylammonium cation obtained in the range of 3.75–30 mM NH_4Ac (90% ACN, ${}^{w}_{w}\text{pH} = 6.0$; see Section 3.3.1). A second-order polynomial fitting was required due to significant curvature of the *k vs.* inverse counter-ion concentration plots.

Maximum values for the ionic interaction contribution, as calculated from measured and extrapolated values of *k* [28], amounted to 92% (benzyltrimethylammonium cation) and 69% (tyramine) and they were obtained at minimal concentration of NH_4Ac and on bare silica (Table 4). An increase of the salt concentration or the ligand surface density each decreased the relative contribution of ionic interactions. This trend was expected, because both processes shield potentially interactive $Si–O^-$ sites. For tyramine, only 5% of ionic contribution remained on the 3.67 μmol m^{-2} bonded packing when using 15 mM NH_4Ac, while this figure amounted to 16% for the benzyltrimethylammonium cation. Due to the fact that under these conditions the absolute retention of tyramine was much higher compared to that of benzyltrimethylammonium cation ($k = 3.56$ *vs.* 1.60) it is justified to conclude that tyramine was much more involved in "other", i.e. HILIC-type, retention mechanisms. Explanation for this comes from fundamental structural differences of these two solutes because apart from the primary amine functionality the phenolic moiety in tyramine is a potential site for hydrogen bonding, while benzyltrimethylammonium cation lacks a polar interactive site that would facilitate penetration into the water-rich layer or that would allow for the development of weak adsorptive interactions with ligand groups.

The observed curvature in the plots of *k vs.* inverse counter-ion concentration was indicative that these HILIC-type interactions superimposed upon the ionic solute–silanol interaction process depended on the counter-ion concentration [28], as follows from the considerations given in Section 4.2.1. In case of using higher concentrations of salt (but also columns having higher ligand density) an increase of HILIC-type interactions compensates for the loss in retention of polar basic solutes resulting from the reduction of ionic solute–silanol interactions. In the present experiments the use of relatively high salt concentrations (max. 30 mM NH_4Ac) even led to an over-compensation for tyramine as demonstrated by "U-shaped" retention curves (Fig. 6b). This effect was more pronounced on bonded packings, possibly because of a lower degree of underlying ionic interactions that would be counteracted by an increased salt load (Table 4). Besides salt concentration, it is clear that the relative contributions of these orthogonal mechanisms can also be controlled by pH variations, type of salt (both affecting protonation and thus compound polarity), temperature, and other elution parameters.

In addition to curved van't Hoff plots, which may reflect different retention mechanisms and changes in phase ratio [40], respectively, it was demonstrated that the retention of tyramine is subject to a smooth transition from an endothermic transfer process on bare silica to an exothermic one on the 3.67 μmol m^{-2} bonded column (Fig. 8 and Table 3). This further substantiated that tyramine undergoes fundamental changes in its retention behaviour depending on the amount of urea-type ligand bonded to the silica surface. HILIC-type interactions are typically characterised by negative values of ΔH [7,25,32], which is in agreement to what was observed for tyramine in case of minimised ionic contribution (i.e. high ligand surface density) and also for non-charged guanosine (Table 3).

Overall, a true 'mixed-mode' retention process composed of solute–silanol cation exchange and HILIC-type interactions was obvious for tyramine. HILIC-type interactions likely strengthen when the solutes are initially driven to the polar interactive sites (water-rich layer, ligand functionalities, surface) via long-ranging ion attraction mechanisms. Such combined/cooperative effects may, adapting the concept of Neue et al. [41], be summarised as "ion exchange assisted HILIC" or "HILIC assisted ion exchange". In the present study, the relative contributions could be effectively balanced via ligand density as well as by elution conditions (cf. Section 3).

The retention of the strongly basic benzyltrimethylammonium cation appeared to be more dominated by cation exchange interactions with the dissociated silanol groups. In case cation exchange sites are shielded by the polar ligand or the analyte gets into

Table 4
Relative contribution (%) of ionic interactions to retention of basic solutes as calculated from k *vs.* $1/[M^+]$ plots (range 3.75–30 mM NH_4Ac for benzyltrimethylammonium cation and 3.75–15 mM NH_4Ac for tyramine) using packings of different ligand surface densities. Mobile phase: ACN/(37.5–300 mM aqueous NH_4Ac, each ${}^{w}_{w}pH = 6.0$) (90:10; v/v). Further chromatographic details are given in Section 2.

NH_4Ac [mM]	Benzyltrimethylammonium cation Ligand surface density [μmol m^{-2}]			Tyramine Ligand surface density [μmol m^{-2}]		
	0	1.81	3.67	0	1.81	3.67
3.75	92 (92)[a]	69 (71)	42 (44)	69	45	25
7.5	87 (88)	59 (61)	28 (31)	56	31	12
15	78 (80)	43 (46)	16 (19)	40	19	5
30	66	29	10	–	–	–

[a] Values in brackets were calculated from plots in the range 3.75–15 mM NH_4Ac.

forced competition with counter-ions (NH_4^+) absolute retention becomes critically low because this solute is not suitable for developing HILIC-type interactions to a significant degree. Thermodynamic data support this interpretation (Section 3.3.5 and Table 3) because retention enthalpies ΔH of benzyltrimethylammonium cation were positive and they decreased with increasing bonding density. Such endothermic transfer processes are in agreement with data from earlier studies involving solutes with prevailing ion exchange interactions under HILIC elution conditions [29,30].

4.2.3. Retention principle of acidic compounds

Acids were more strongly retained on more densely bonded packings and when using higher salt concentrations, with respective k values responding more strongly to changes of these parameters compared to the selected non-charged compounds. Explaining these retention trends of acidic solutes exclusively by HILIC-type interactions is contradicted by data on absolute retention. For example, the highly polar 4-hydroxybenzenesulfonic acid (calculated log $D^{pH\,4.0} = -5.10$) was retained much more weakly compared to the less polar compound guanosine (log $D^{pH\,4.0} = -1.72$) and others (90% ACN, 10 mM NH_4FA, ${}^{w}_{w}pH = 4.0$). Moreover, 4-hydroxybenzoic acid (calculated pK_a value 4.57 at pH = 4.0; not considering potential shifts in hydro-organic environment) virtually co-eluted with 4-hydroxybenzenesulfonic acid (calculated pK_a value −0.23) under these elution conditions on bare silica, despite the much lower polarity of the carboxylic acid (log $D^{pH\,4.0} = 1.32$). At ${}^{w}_{w}pH = 7.0$ retention of 4-hydroxybenzoic acid on bare silica was even higher than that of 4-hydroxybenzenesulfonic acid, plausibly explained by a gain in compound polarity of 4-hydroxybenzoic acid due to deprotonation. Still, however, the log D value of 4-hydroxybenzoic acid was much lower (log $D^{pH\,7.0} = -0.94$) than that of 4-hydroxybenzenesulfonic acid (log $D^{pH\,7.0} = -5.18$). An inverted elution order could only be observed when using the 3.67 μmol m^{-2} bonded packing. Log D values thus failed severely in estimating differences in the retention of acids (and frequently also of bases; *data not shown*), especially on bare silica. This is in contrast to what was observed for non-charged compounds. Thus, a HILIC-type interaction process cannot be solely responsible for the retention of acidic compounds on the investigated packings; rather, superimposed repulsive ionic solute–silanol interactions are a plausible explanatory model.

Repulsive interactions cause ion exclusion from the stationary phase thereby acting detrimentally to HILIC-type interactions. In the event of packings having a net negative surface charge anionic analytes are driven away from potential interactive sites (water-rich layer, ligand functionalities, surface silanols). Keeping elution conditions constant but increasing the density of the non-chargeable urea-type ligand helped to reduce these repulsive interactions because of a reduction of interactive surface silanols. Consequently, the amount of salt contained in the eluent titrated residual surface silanols to a greater extent on such more densely bonded phases. This is reflected by thermodynamic data because the large difference in ΔH values of 4-hydroxybenzenesulfonic acid obtained on bare silica and the 3.67 μmol m^{-2} packing (Table 3), especially in comparison to those of non-charged guanosine, indicates the presence of superimposed interactive mechanisms that change profoundly in their relative contribution as a function of ligand density.

The progressive shielding of acidic silanol groups by higher concentrations of salt cations is supposed to be a more defined stoichiometric process than the effects of salt on HILIC-type interactions. This explains the gain in retention of 4-hydroxybenzenesulfonic acid (Fig. 6c) already evident at very low salt concentrations and increasing steadily with salt concentration, in contrast with the trend found for non-charged compounds (guanosine in Fig. 6d).

The extent of electrostatic repulsion for acidic compounds on packings with a negative surface charge could also be reduced by using buffers of low pH. As discussed in Section 4.2.1, among the types of salts studied the use of NH_4F_3Ac was supposed to be most effective in suppressing silanol ionisation. This was confirmed with 4-hydroxybenzenesulfonic acid, for which the use of NH_4F_3Ac was quite an effective means to increase retention (Fig. 7a). Solute polarity was obviously not affected because the strong acid was still fully deprotonated under these conditions. By contrast, for 4-hydroxybenzoic acid (Fig. 7c) lowest k values were obtained with NH_4F_3Ac, being explained by a large degree of protonation of the carboxylic acid group which reduces analyte polarity and thus weakens HILIC-type interactions (pK_a value in a similar range as silanols).

A subtle effect of the type of salt is exemplified with the retention trend of 4-hydroxybenzoic acid when using TEAAc as eluent additive. Under such conditions 4-hydroxybenzoic acid eluted earlier on more densely bonded packings. This stands in sharp contrast to what was observed with NH_4Ac, NH_4FA, and NH_4F_3Ac. At low ligand surface coverage virtually the same values of $k^{4\text{-hydroxybenzoic acid}}$ were obtained on the bare silica column (Fig. 7c). Under such conditions the salt cations (NH_4^+ and TEA^+) primarily reduce electrostatic repulsion by forming ion pairs with surface silanols. Upon increasing the ligand surface coverage the fraction of surface silanols decreases which allows a larger fraction of salt cations to interact with the acidic solute. The ion pair formed with NH_4^+ is more hydrophilic compared to the ion pair formed with TEA^+. This is tantamount to a decrease in retention of the TEA^+-ion pair at higher ligand surface coverage. On the other hand, for 4-hydroxybenzenesulfonic acid (Fig. 7a) shielding of electrostatic repulsion is much more important to increase retention compared to 4-hydroxybenzoic acid and this explains why for 4-hydroxybenzenesulfonic acid the use of TEAAc led to an increase in retention analogously to the other salt additives.

In addition to what was found for other types of solutes (Section 3.3.2) also the data of acidic compounds emphasise that the choice of the type of salt may have a decisive effect on the separation process under HILIC elution conditions, whereby these effects have to be understood as actually being integrated salt/pH-effects.

It may be concluded that the retention of acidic compounds on bare silica and the home-made urea-type packings is generated by HILIC-type mechanisms. Ionic solute–silanol interactions counter these processes and lead to repulsion effects. Such a 'mixed-mode' electrostatic repulsion/hydrophilic interaction retention behaviour, controlled by the analysis conditions (especially by parameters affecting charge state of chromatographic material and analyte), was recently proposed by Alpert as a complementary concept (so-called "ERLIC" mode) for the isolation of phosphopeptides [19].

4.2.4. Retention principle of amphoteric compounds

From the mechanistic interpretations drawn for non-charged, basic, and acidic compounds it becomes clear that also the retention of amphoteric solutes under HILIC elution conditions may be a rather complex event. The intrinsic high polarity of zwitterions makes them particularly suitable for HILIC-type interactions. In case attractive ionic interactions with the stationary phase are made possible by the analysis conditions, multiplicative effects such as discussed for bases (Section 4.2.2) may lead to extremely high retention. For example, acetylcarnitine exhibited a k value of 122 on bare silica (elution conditions according to Table 2) while the retention of all other solutes given in Fig. 2 was much weaker ($k < 19$).

The retention of tyrosine appeared to be strongly determined by HILIC-type interactions because its absolute retention was significantly higher compared to that of its "building blocks" tyramine and 4-hydroxybenzoic acid (Fig. 4). The distinct "U-shaped" retention trend of tyrosine observed at ${}^{w}_{w}\mathrm{pH} = 6.0$ on packings having different ligand surface densities (Fig. 4c) is ascribed to a dual process. On bare silica the retention of tyrosine in its zwitterionic state is described to be generated by a combination of HILIC-type and attractive ionic interactions. Repulsive interactions between carboxylate group and silanols may be present as well. Introduction of the ligand to the surface weakens the electrostatic attraction component more than the electrostatic repulsion component. The result is a net decrease in retention. When ligand surface coverage reaches 1.81 μmol m^{-2} the increase in HILIC-type interactions comes to dominate the chromatography and retention increases again. Opposed to that, the lower polarity of tyramine at ${}^{w}_{w}\mathrm{pH} = 6.0$ as well as the lack of a carboxylate functionality accounts for the lack of a U-shaped curve for this solute because the decrease in electrostatic attraction by the basic group outweighs the increase in HILIC-type retention through conversion of silanols to the bonded ligands (Fig. 4b).

As exemplified in Table 2 for the selectivity of tyrosine/4-hydroxybenzoic acid in comparison to tyramine/4-hydroxybenzoic acid, variations in elution conditions targeted at changes in the overall charge state of zwitterions, and especially the pH value, may exert more profound effects on retention and selectivity compared to bases, acids, and also non-charged compounds. This again shows the great optimisation potential that may be available during HILIC method development.

5. Concluding remarks

The present study focussed on gaining insight in the retention mechanisms at work under HILIC elution conditions on bare silica and on bonded packings of different ligand surface density. Although a structurally simple and non-chargeable ligand was chosen, rather complex processes that generate retention and selectivity for non-charged compounds, bases, acids, and amphoteric compounds were revealed. Apart from studies on ligand surface coverage, the remarkable effects generated by variations of elution conditions signified the great optimisation potential for retention and selectivity residing in mobile phase composition and temperature. A point-by-point evaluation of individual elution parameters for adjusting retention and selectivity was beyond the scope of the present study. Nevertheless, it was found that in particular the type of salt and its concentration in the hydro-organic eluent seems to influence the separation processes in multiple ways. This aspect deserves further investigations.

As long as solute–silanol interactions are not sufficiently suppressed by the selected analysis conditions, 'mixed-mode' separations for charged solutes seem to be the rule and not the exception on bare silica as well as on the selected urea-type packings. HILIC-type interactions themselves are also regarded as having certain 'mixed-mode' character, because there likely exists an orthogonality between partitioning and (the more selective and flexibly adjustable) weak adsorptive interaction processes. The mechanistic situation may be further complicated in case of using packings with immobilised chargeable ligands.

The higher selectivity values obtained on bare silica for compound pairs with different charge state emphasised high orthogonality of ionic mechanisms to HILIC-type interactions. On the other hand, molecular distinction prevailingly based on HILIC-type interactions (i.e. separation of non-charged compounds or compounds with the same charge state but different non-charged functionalities) was improved when using bonded packings or elution conditions favouring HILIC-type interactions (especially higher salt concentration), cf. Tables 2 and 3.

Standardised column classification tests would greatly help to assess the separation properties of different types of polar columns. The present study demonstrated that even a compound set that is quite small, composed of solutes of very different charge states, allows pinning down differences in separation properties solely arising due to variations of ligand bonding density and the surface silanol fraction, respectively (Fig. 10). The chemometric dissimilarities between urea-type and the various amide-type packings can, however, not be discussed on a valid mechanistic basis until details on the physico-chemical properties of the commercial columns become available. Nevertheless, the significance of the composition of the packing material has been elucidated and its role under HILIC elution conditions is certainly not limited to that of a polar backbone upon which a water-enriched layer is generated [28].

There is a need to further characterise the plethora of retention processes and selectivity increments that may be accessible under HILIC elution conditions. An improved mechanistic understanding will form the basis for the efficient development and optimisation of HILIC methods in daily practice.

Acknowledgements

The financial support of the Christian Doppler Research Society and the industry partners Merck KGaA (Darmstadt, Germany), AstraZeneca (Mölndal, Sweden), and Sandoz (Kundl, Austria) is gratefully acknowledged. The authors are indebted to Dr. Uwe Neue (Waters Corporation, Milford, MA, USA) for generous provision of the XBridge Amide column and to Dr. Yi Chen (State Key Laboratory of Food Science and Technology, Nanchang University, China) for help with chemometric calculations.

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