DISSERTATION

Development and evaluation of new silica based polar and / or mixed modal stationary phases for HPLC

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1. Introduction

Since the origin of high-performance liquid chromatography (HPLC) in the 1960s, there has been a continuous development of stationary phases for columns. First columns in HPLC (1964-1965) were dry-packed with diatomaceous earth particles of about 100 μm into glass or metal tubes that were largely used for gas chromatography (GC). In the demand for high column efficiency columns packed with smaller particles were developed and the suitable packing technique such as 'tap-fill' and slurry packing methods were designed as well. The developments of bonded phase columns in early 1970s expanded greatly the application of HPLC in separation science area. Now HPLC has become a mature separation technique and it is an indispensable tool to deal with complex samples as for (bio)analytical problems. HPLC is utilized for both routine applications and research in fields like pharmaceutical sciences, bioanalytics, biomedical sciences and environmental sciences. Before introducing a few available HPLC separation modes, support materials of stationary phases for HPLC will be reminded firstly.

1.1 Support materials

Most stationary phases are based on silica while the use of polymeric supports was reported as well. Silica gel consists of small and spherical particles with amorphous structure. For analytical HPLC applications particle diameters typically range from 1.7-5 μm. Silica gel is highly porous with various pore sizes from 60-4000 Å of narrow distribution resulting in quite different active surface area. Silica gel is composed of hydrophobic siloxane and hydrophilic silanol groups present in single, geminal or vicinal form on the surface and have good mechanical strength. The exposed silanol groups on the surface of silica present the main adsorption sites of silica and are also the most active functions which can be easily modified with organic moieties for desired applications [1, 2]. Another generation of silica-based stationary phase are monolithic silica phases. Monolithic silica columns have much higher porosity than the conventional silica particle packed columns and it can be considered a single piece of highly porous silica with bimodal connected pore system of macropores and mesopores. The macropores form a

network of pores with an interconnected flow path (through-pores) and the mesopores form the fine porous structure of the column. Monolithic silica columns are characterised by the high separation efficiency and fast separation speed without large backpressure due to the high porosity [3]. However, the preparation of monolithic silica columns is in the dimensions of capillaries to 2 mm ID format not so easy and there may be a problem for reproducibility [4].

Polymeric supports such as polystyrene divinylbenzene polymer (PS-DVB) and polyvinyl pyridine-polystyrene polymer (PV-PPS) can be used to manufacture stationary phases for HPLC and there is a wide variety of commercially available polymeric supports. Polymer-based stationary phases do not show peak tailing problems for basic compounds owning to the silanol groups as that for silica-based stationary phases. Polymer-based phases possess high chemical stability up to pH 14. However, polymer supports are not as popular as silica gel owing to the difficult preparation methods of polymer and lower column efficiencies etc. [1]. Nevertheless, capillary type columns of this type become popular in proteomics and protein separations.

1.2 Normal-phase liquid chromatography (NPLC)

NPLC is one of the first kinds of available HPLC separation mode. NPLC is typically performed with polar stationary phases such as bare silica, diol-, amino-, cyano-bonded phase and less polar mobile phase. The eluents in NPLC is usually a mixture of organic solvents containing a non-polar solvent such as hexane, heptane and a more polar solvent such as chloroform and alcohols etc. in which the polar solvent molecules will be adsorbed on the polar sorbent surface.

In NPLC, the retention is considered to be a result of competitive adsorption between the solute molecules and the strongly eluting solvent molecules for the adsorptive sites on the adsorbent surface and it is increased when the phase polarity increases. When the retention is discussed in NPLC, a few important factors have to be considered, namely (i) the polar substituents in the solute molecular and its molecular area, (ii) so-called secondary solvent effects referring to the solute-solvent interactions in both the mobile and adsorbed phases which could cause useful changes in retention and even new chromatographic selectivity,

(iii) the potential localization of polar molecular with respect to both solutes and solvents and the preferential adsorption of solutes and solvents on polar sites of the ligands [5].
NPLC has some practical advantages. For example, organic solvents offer a low viscosity and thus low column flow resistance. In addition, bonded stationary phases are more chemically stable in organic solvent than in aqueous-organic mobile phase. NPLC is suitable for the separations of moderately polar compounds while it is not useful for highly polar compounds due to their poor solubility in the non-polar eluents and the very high retention factors.

1.3 Reversed-phase liquid chromatography (RPLC)

Among the separation modes available in HPLC such as NPLC, reversed-phase liquid chromatography (RPLC), ion exchange chromatography (IEC) or size exclusion chromatography (SEC), RPLC is the most frequently used one. The dominant position of RPLC is due to its suitability for separating a wide range of analytes ranging from small to large molecules as well as from non-polar to intermediate-polar compounds. RP-type stationary phases are usually based on porous silica particles which are surface modified by chemical reactions. RP-type chromatographic ligands typically consist of alkyl chains like C_8, C_{18} but sometimes also of a hydrocarbon polymer layer which are covalently bonded to the support surface. The retention of analytes in RPLC is fundamentally determined by their distribution between a polar mobile phase (mixtures of water and organic modifiers) and the less polar stationary phase. As a rule of thumb solutes are eluted in order of increasing hydrophobicity. These surface-bonded alkyl chains in RP columns can be highly solvated by components of the mobile phase which means they can selectively adsorb components of the eluents resulting in a formation of a thick solid-liquid interface with a complex structure. The retention in RPLC was first proposed to be a partitioning mechanism of solutes between the hydroorganic mobile phase and this quasi-liquid layer. Depending on the nature of the mobile phase, two configurations of the stationary phase could occur. In organic solvent-rich eluents, the bonded alkyl chains can be extended as a fur-like configuration where the solute molecules can associate with the tips of the alkyl chains or penetrate into this layer and interact with the lateral surface of

ligands. In water-rich eluents, the bonded alkyl chains have closer contact with each other leading to a collapsed structure where the solute molecules probably have less contact area with the bonded chains resulting in a earlier elution. If the ligand density is small enough, the bonded chains can be considered as isolated solvated chains. In other words, if the ligand density is high enough, they interact among themselves and a penetration of solute molecules into this amorphous hydrocarbon layer would be disallowed. In this case, the retention is probably governed by the surface adsorption. The retention in RPLC is dependent on surface coverage of ligand, the length of the alkyl chains, the surface tension of mobile phase, and the molecule size of analytes as well and it can not be ascribed to either partitioning interactions or adsorptive interactions alone [6, 7].

Conventional silica-based RP-type columns have limited chemical stability at extreme pH value (pH < 3 and pH > 8) and a relatively low thermal stability up to 60°C. The chemical and thermal stability has been much improved by (i) the development of new starting substrate materials such as hybrid organic-inorganic silica which contains SiO_2 as well as $R(Si)_{1.5}$ groups (R, methyl or ethyl) leading to a decreased hydrolysis, (ii) the new ligand attachment like bidentate modified silica in which the ligand is bonded on the surface by a dual bond from two different silicon atoms in the ligand, (iii) the sterically protected silica which contains isopropyl side groups instead of methyl side groups in conventional RP phases leading to a decreased interactions between residual silanols and the ligand siloxane oxygen atom, and (iv) the coating of polymer on the silica surface. Until now, many RP-type columns are chemically stable with a wider pH range from 1 to 12 and thermally up to 100°C [8, 9]. Nevertheless, RP columns with even higher chemical and thermal stability are still required owning to the demand for faster method development, more quickly analysis, and further improved selectivity. Another critical problem in RPLC is the broad and tailing peaks of basic compounds on silica-based phases which can affect resolution, sensitivity and reproducibility. This is induced by the unwanted secondary interactions such as ion exchange, hydrogen bonding and dipole-dipole interaction between the free or charged residual surface silanol groups and the basic sites of solutes. There are many different approaches that have been used to block the remaining silanols such as (i) silanol endcapping by a trimethylsilylation reducing the access to free silanols, (ii) adding organic additives to mobile phase like triethylamine competing with basic solutes for interaction with free silanols, (iii) altering the pH value of eluents reasonably to change the ionization

conditions of silanol groups or basic solutes reducing their ion exchange interactions [10]. Though that, the residual surface silanols usually can be reduced but not completely eliminated.

However, major shortcomings of RP columns reside in the fact that polar compounds such as sugars, amino acids and nucleosides which are relevant components in different fields of the life science such as proteomics, metabolomics, and pharmacology etc. can not or not sufficiently be retained. In order to retain such polar compounds on classical C_8 or C_{18} columns, usually a high percentage of water in the mobile phase or pure water is required [11]. As aforementioned, under such highly aqueous elution conditions the solvation of the alkyl chain will be suppressed (de-wetting phenomenon) followed by a phase collapse *i.e.* ‘‘ligand deactivation’’ resulting in weaker and non-reproducible retention data.

Many attempts have been made to remedy this drawback of RPLC. Various new approaches of silica phase modification, such as polar embedded chromatographic ligands (e.g. amide group within the alkyl ligand) or polar endcapped phase (e.g. hydroxy groups immobilised on the surface of silica in addition to the alkyl chains), have been developed to improve the wetting (solvation)-conditions of stationary phase using highly aqueous eluents [12, 13]. Compared to conventional C_{18} columns, these polar embedded- and polar endcapped stationary phases are characterised by an improved chemical stability under highly aqueous conditions, improved peak shape for basic compounds proposed due to a more effective shielding of residual surface silanols or a competitive interaction of the embedded groups with silanols and unique selectivity pattern owning to some extra interactions such as dipole interaction with nitrogen-containing embedded groups [13, 14]. However, according to literature, these polar embedded- and polar endcapped phases provide sometimes reduced retention for basic compounds than conventional RP phases [12]. Probably this is a result of some specific interactions at the presence of the embedded groups in ligands. Yet, the retention of highly polar compounds such as amino acids and sugars is still very low on such polar-modified columns. Apart from this chromatographic aspect, it is as well noted that the use of highly aqueous elution conditions may also cause sensitivity problems in mass spectrometric (MS) detection due to an increased bleeding of the phase caused by an increased incidence of hydrolisation of silans.

1.4 Hydrophilic Interaction Chromatography (HILIC)

1.4.1 Introduction

Owing to the growing demand of analysis of highly polar compounds in *life science* fields, the development, characterisation, and application of alternative types of stationary phases are therefore desirable in order to end up with more suitable separation conditions for such compounds. As an alternative elution mode to RPLC the so-called Hydrophilic Interaction Chromatography (HILIC) attracted great interest in the past few years. HILIC is a separation technique which is targeted at generating retention especially for highly polar compounds. The acronym HILIC was first introduced in 1990 by Alpert [15], but HILIC-like separations are already in use since the 1970s [16]. HILIC can be characterized as a variant of NPLC and it also employs polar packing (charged or non-charged) such as amino-, amide-, diol-, zwitterionic silica etc.. In the HILIC elution mode, the mobile phase is a mixture of an organic solvent (typically acetonitrile (ACN), mostly 60-95%, v/v) and a protic solvent (usually water, as the strong eluting modifier) with volatile salts such as ammonium acetate, or ammonium formate. The use of water in eluents makes better solubility of highly polar analytes in HILIC eluents than in that of NPLC and the high amount of organic solvent in eluents offers the possibility to use high flow rate of mobile phase without high column backpressure [15, 17, 18].

1.4.2 Retention mechanism

In HILIC, it is commonly believed that water molecules are being attracted by polar groups on the stationary phase (e.g. surface silanols, ligand functionalities) to form a quasi-stagnant layer (Figure 1). The retention of polar compounds can then be imagined to occur via a differential distribution of analytes between the water-enriched layer and the less-polar bulk mobile phase, which is tantamount to a partitioning mechanism. Thus, solutes are basically retained and separated according to their different polarities.

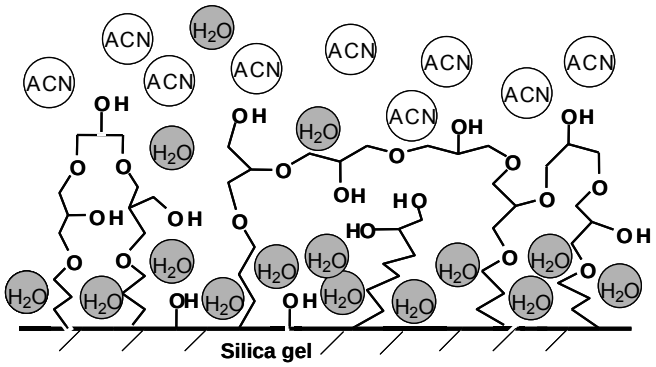


Figure 1. Schema of the micro environment around the surface of a polar stationary phase (exemplified with hydroxyl-type modified silica) in case of using a mobile phase rich in ACN

This partitioning mechanism for HILIC retention was supposed in early 1980s and was based on (i) a decrease of retention as the difference of phase polarity between two phases was decreased, (ii) an increase of retention with increasing number of polar groups in the bonded moiety which in turn caused an increasing amount of stationary eluents in the pores, (iii) the unchanged ratios of retention factors of all the investigated solutes among the columns which excluded the phase selective interactions [18].

More recently, McCalley and Neue examined whether the water-rich layer actually exists on the surface within the pores of bare silica when using water-deficient mobile phases. This was accomplished by measuring the retention of benzene and toluene as a function of water content in aqueous-ACN mobile phases on a superficially porous bare silica column [19]. A U-shape relationship between the retention times of benzene/toluene and % water v/v in the mobile phase was obtained and the minimum retention was observed around 30% water (v/v). The initial decrease in retention with low content of water (< 30%, v/v) in eluents was hypothesised to be due to the establishment of a water-rich layer on the silica surface. By increasing the thickness of the water layer, *i.e.* using mobile phases with higher water content, the retention time of benzene and toluene was decreased due to an exclusion effect from the water rich pores of the phase. However, upon increase of the water fraction in the eluent to > 30% (v/v) polarity differences between the aqueous surface layer and the flowing mobile phase started to decrease and, consequently, benzene and toluene molecules started partitioning into the surface layer due to a hydrophobic retention mechanism exerted by the relatively hydrophobic siloxane groups deriving from the modified silica. The presence of water-rich layer on the surface of stationary phase in

HILIC elution mode is strongly supporting the involvement of the aforementioned partitioning retention mechanism.

Over the past few years, more and more experimental data were reported which led to the conclusion that a simple partitioning-based model is often not sufficient to explain retention in the HILIC elution mode [18]. Those arguments about the deviation of HILIC retention from the early declared pure partitioning mechanism are mainly based on two basic equations which describe

(i) the pure partitioning interactions (derived from RP chromatography):

$\log k'(\varphi) = \log k_w' - S.\varphi$

(k_w' is the retention factor of the weaker eluent as mobile phase, φ is the volume fraction of the stronger eluent of a binary mobile phase, S is the slope of linear relationship of log k' versus φ plot) and (ii) the pure adsorptive interactions (derived from NP chromatography):

$\log k'(\varphi) = \log k_B' - S'.\log \varphi$

(k_B' is solute retention factor of pure B as eluent, S' is equal to –d[log k']/d[logφ] for a give analyte and polar solvent, φ is the volume fraction of the stronger eluent of a binary mobile phase).

A linear relationship of log *k*' *versus* water volume fraction in eluent (lin-log plot) or log *k*' *versus* log (function of water volume fraction in the eluent) (log-log plot) can thereby be taken as an indication whether partitioning or adsorptive interactions are the driving retention mechanism.

For example, a linear relationship of log-log plots of a set of peptides was found under HILIC elution conditions when the water content in the eluent was varied between 3 and 45% (v/v) with 0.1% TFA in ACN on TSK gel Amide-80 column and all correlation coefficients r were greater than 0.99 [20] and it was concluded that the adsorptive interactions were involved in the retention mechanism on this column. Actually, the involvement of adsorptive forces such as dipole-dipole interactions in HILIC retention was earlier mentioned cautiously by Alpert, but no experimental evidence was given to support this assumption [15].

Berthod et al. suggested that the retention mechanism of sugars on cyclodextrin-bonded stationary phases (CDs) in the HILIC elution mode is intermediate between partitioning and adsorption [21]. Support for a partitioning mechanisms was provided by (i) the linear proportional relationship of log *k*' of a few oligosaccharides as a function of their degree of polymerization (DP) ranging from 1 to 6 with regression coefficients over 0.992 which is

commonly observed in RPLC and the decrease in slope with increasing of water content in mobile phase and (ii) the linearity of the logarithm of the energy $\Delta G^0_s/RT$ *versus* % of water in eluents (*according to the equations: log k' = $\Delta G^0/RT$ + log Φ and log k' = $\Delta G^0_{ms}/RT$ + (DP-1) $\Delta G^0_s/RT$ + log Φ*) and the slopes of plots were approximately identical within experimental errors for all oligosaccharides. On the other hand, (i) the non-linear relationship of log *k*' *versus* % of water in eluents (lin-log plot) and (ii) the extremely long retention time of ''600 million years'' under 100% ACN elution conditions, which was extrapolated from the relationship of log $\Delta G^0_s/RT$ *versus* % water in eluents, were taken as argument for hydrogen bonding interactions at low water content and thus adsorptive forces.

Many experimental studies tried to elucidate the retention mechanism in the HILIC elution mode and it seems that a mixed retention model composed of partitioning and adsorptive increment gains acceptance. Besides hydrophilic interactions (partition and adsorption), superimposed ionic interactions between charged solutes and charged ligands or ionized residual surface silanol groups were observed in HILIC elution mode and these electrostatic interactions have a strong effect on retention [22, 23]. Such mixed-mode hydrophilic interaction/ionic exchange chromatography has been proved to be particularly useful for separation of highly charged peptides and proteins [24-26]. In this case, a hydrophilic ion-exchanger is employed as stationary phase and a high amount of organic solvent (generally ACN) is used to promote ''hydrophilic interactions'' between solutes and stationary phase. The separations are based on two different separation mechanisms, namely on hydrophilicity/hydrphobicity and on net charge of solutes where solutes are eluted in groups in order of increasing net charge and within these groups solutes are eluted in order of increasing hydrophilicity, respectively. The simultaneous presence of different mechanisms provides increased chromatographic selectivity compared to a single-mode approach [27]. Recently, Alpert has put a so-called electrostatic repulsion hydrophilic interaction chromatography (ERLIC) forward [27]. It is based on a weak anion exchanger column for separation of basic and of acidic peptides as well of phosphopeptides whereby the column was operated under HILIC elution conditions. In ERLIC, acidic peptides can be retained on the column through electrostatic attraction (ion exchange) and basic peptides can be eluted in the elution time frame of neutral or slightly acidic peptides through the balance of electrostatic repulsion and hydrophilic interactions.

This makes an isocratic resolution of mixtures such as heterogeneous peptides, amino acids and nucleosides etc. possible which is convenient for method development.

Within the framework of hydrophilic/ion-exchange mixed-mode chromatography, another column variant is the use of hydrophobic/ion-exchangers (so-called RP/WAX materials) in the HILIC elution mode. RP/WAX ligands contain an alkyl chain for hydrophobic interactions and a cationic site for anion-exchange [28]. Basically, the combination of orthogonal retention mechanism hydrophobic and ionic interaction offers multimodal applicability in the RP elution mode. However, due to the presence of some polar functionalities in RP/WAX ligands (ion exchanger site, embedded amide group) hydrophilic and ionic interactions can be exploited in the HILIC elution mode as well [25, 29, 30]. Thus, such mixed-mode phases may provide complementary selectivity patterns to other HILIC stationary phases available on the market.

Another particularly noteworthy point in the HILIC elution mode is the silanol activity when the analysis is based on silica-based stationary phases. Although the bonding of ligands blocks the silanol groups partially, there are still some silanol groups available on the surface of silica. Most commercially available HILIC phases are recommended to use volatile salts to suppress the possibly occurred electrostatic interactions between charged solutes and ionized surface residual silanol groups of silica-based stationary phase. Silanol-related interactions are well known to affect the separation properties of RP packing but with respect to HILIC phases their influence has not yet been studied adequately.

A wide range of compounds including proteins, peptides, amino acids, oligonucleotides, carbohydrates as well as other metabolites, drugs, and toxins is analysed by HILIC [15, 24, 31-40]. This wide application is related to some properties of the HILIC separation technique. For example, the high content of ACN in HILIC eluents offers HILIC very good compatibility to MS detection, especially with respect to the more efficient evaporation of solvent which is beneficial to detection sensitivity [17, 41-44]. In addition, faster separation can be achieved in HILIC mode using high flow rate of mobile phase thereby less sacrificing peak efficiency and leading to lower column backpressure compared to RPLC [17, 43, 45]. Furthermore, HILIC and RPLC are of high orthogonality both in operation technique and also in retention mechanism which leads to quite different elution orders [15, 24, 31, 46]. In other words, the HILIC elution mode is often best suitable for compounds that are troublesome to retain in RPLC. Consequently, the high orthogonality

of HILIC and RP selectivity patterns [47, 48] is very attractive for multi-column approaches (on-line coupling or off-line analysis on both RP and HILIC columns) to broaden the compound window accessible in a single chromatographic run in applications involving complex samples such as Traditional Chinese medicine preparations or pharmaceutical impurity profiling [46, 49-51].

Associated with the wide application of HILIC, a number of polar stationary phases have been made commercially available in the past years, respectively. In the following section the most important types of polar stationary phases are introduced with respect to their surface chemistry, their separation characteristics, and their typical fields of applications.

1.4.3 Stationary phases applicable for HILIC separations

1.4.3.1 Bare silica

Bare silica, underivatized silica gel, is a classical packing material originally used in NPLC. The silanol groups on the surface of silica (Figure 2) are considered as hydrophilic sites and different types of silanol have different acidity and reactivity [2, 52]. Silanol groups may exert strong polar interactions in the HILIC elution mode. They can attract water or protic modifier molecules by hydrogen bonding from mobile phase which leads to the formation of aqueous layer on the surface of the sorbent. The retention preocess on bare silica could be complicated involving hydrophilic interaction, ion-exchange and some hydrophobic retention by the siloxane backbone depending on the pH value of mobile phase, particularly for charged or chargeable compounds [17]. Many commercially available bare silica columns such as Atlantis HILIC (Waters, Massachusetts, USA), Hypersil, Betasil (both Thermo Scientific, Karlsruhe, Germany), Kromasil (EKA Chemicals, Göteborg, Sweden) etc. were already employed for HILIC separations [17, 31, 53-55].

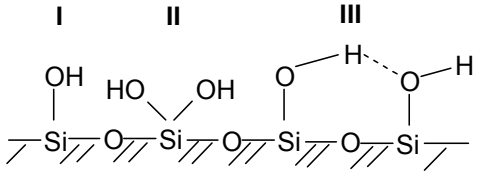


Figure 2. Different types of silanol group on the surface of silica: I. Free silanols; II. Geminal silanols; III. Vicinal silanols

Silica gels from different manufacturers contain different amounts of silanol groups on the surface, different ratio of the different types of silanol groups and different purities [31] which can have an effect on the point of zero charge of silanol groups at which the silanols are unassociated [2]. Usually it is assumed that at pH 4.0 in water all silanol groups are fully protonated and above pH 4.0 the silanol groups start to ionize. So there is potential for electrostatic interactions beside the hydrophilic interactions on bare silica which is dependent on pH value of mobile phase [22].

For liquid chromatography-tandem amss spectrometry (LC-MS/MS), underivatized silica packing can be much more attractive than bonded silica columns on which the bonded organic moieties could be released from the surface and then interfere with the MS-based detection (so-called column bleeding) [17]. However, a major drawback of using bare silica is the occurrence of irreversible adsorptions of analytes [53] and they possess limited chemical stability in basic elution conditions, typically pH >8, leading to dissolution of silica skeleton [1, 56, 57].

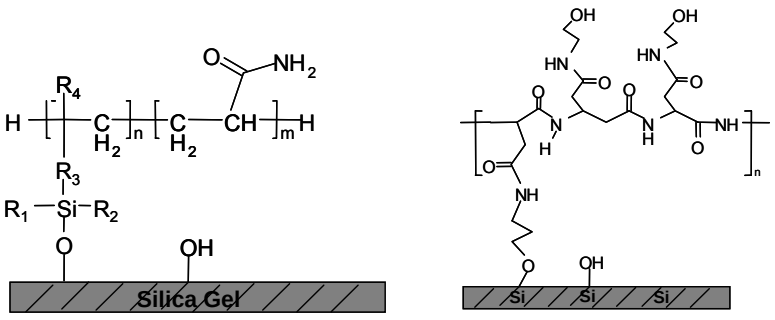
In HILIC mode, bare silica columns are mostly utilized for separation of small polar compounds such as acetylcholine, choline, nicotinic acid, metabolites in human plasma and nucleobases etc. while applications for separation of carbohydrates, peptides or proteins are limited [58].

1.4.3.2 Non-charged stationary phase

1.4.3.2.1 Amide- and poly(2-hydroxyethyl) aspartamide stationary phase

Amide-bonded silica like TSK Gel Amide-80 (Tosoh, Tokyo, Japan) is one of the most popular stationary phases to be used in the HILIC elution mode. According to the manufacture, amide silica contains a covalently bonded carbamoyl functional group on the surface of silica gel via a short aliphatic spacer (Figure 3a). Amide groups in amide silica have lower chemical reactivity compared to the amino groups in amino silica and they do not offer ion-exchange capabilities in the usual pH working range [22]. Theoretically, amide silica could still have some electrostatic interaction property due to the negatively charged surface residual silanol groups under certain conditions [59]. However, this electrostatic interaction on amide phase should be much weaker than those on bare silica

phase. Amide silica does not react with reducing sugars forming schiff base and it is suitable for separation of carbohydrates [60, 61]. According to literature, amide-type stationary phases have just limited access to irreversible adsorption and they provide good stability for HILIC separation even after 500 injections of peptides which is superior over amino-bonded silica [61, 62]. Separations of monosaccharides, oligosaccharides, derivatized carbohydrates, peptides, and amino acids etc. are reported on amide phases [20, 22, 59, 61-64].



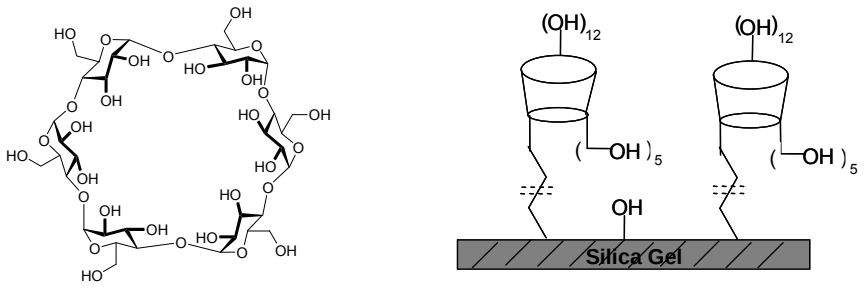
(a) (b)

Figure 3. The chemical structure of functional groups of amide silica (a) and poly(2-hydroxyethyl) aspartamide stationary phase (b)

Based on aminopropyl silica a poly(succinimide) silica is prepared by immobilisation of poly(succinimide). A Poly(2-hydroxyethyl) aspartamide material (Figure 3b), so-called PolyHydroxyethyl A (PolyLC, Columbia, MD, USA) can be obtained through an aminolysis of poly(succinimide) silica with ethanolamine [15]. PolyHydroxyethyl A is a quite hydrophilic packing which lacks charged character in principle while in fact there is still ion exchange in certain degree due to the incompletely modified residual amino groups in background. This phase was particularly designed for HILIC application and has been used for analysis of peptides, amino acids, nucleotides, nucleic acids, oligonucleotides and melabolite in plant etc. [15, 27, 35, 65].

1.4.3.2.2 Cyclodextrin-modified silica

Cyclodextrin-modified stationary phases possess cyclic oligosaccharides composed of six- (α-CD), seven- (β-CD), or eight- (γ-CD) α 1-4 linkages of D-glucose units on the surface (Figure 4a) and the whole CDs is a toroid structure (Figure 4b).



(a) **(b)**

Figure 4. Structure of α-cyclodextrin molecular (a) and α-cyclodextrin-based silica (b)

CDs contain relatively hydrophobic cavities (the interior of the toroid structures) which can host hydrophobic molecules by formation of inclusion compounds and this can be exploited in RP mode separation as well [66]. In contrast, the exterior of the toroid are hydrophilic hydroxyl groups in high density which can adsorb water molecules from hydro-organic eluents and form an aqueous layer on the exterior face of CDs phase by hydrogen bonding. This makes such phases conceptually applicable for HILIC-type separations as well. Furthermore, the optically active sugars in cavities of CDs offer this stationary phase chiral separation capabilities and CD packings have successfully been used for the separation of some chiral pharmaceutical compounds in the HILIC mode [67, 68]. The multi-modal chromatographic properties (RPLC/HILIC) of CDs as well as their chiral separation capabilities provide a large degree of flexibility in HPLC method development and these phases hold promise for application in multi-dimensional separations.

In HILIC mode, cyclodextrin-bonded silica has been applied to separate nucleosides, organic acids, alkaloids, phosphorylated carbohydrates, mono-, oligosaccharides such as fructose, sucrose, lactose, arabinosides, celloside, cyclodextrins and maltosides as well as xyloside etc. [21, 66, 67, 69, 70].

1.4.3.2.3 Diol-modified stationary phase

Diol-bonded stationary phases are concerned to be non-charged chromatographic materials (Figure 5) except the potentially negatively charged residual surface silanol groups. Diol silica have for example been commercialised as Inertsil Diol (GL Sciences, Tokyo, Japan), LiChrospher 100 Diol (Merck, Darmstadt, Germany), ProntoSil Diol (Bischoff

Chromatography, Leonberg, Germany) and Luna HILIC (Phenomenex, Torrance, CA, USA). Generally, diol-bonded silica can be used in NPLC with hydrophobic mobile phase like hexane/isopropanol or hexane/methanol for separation of polar compounds such as purines, pyrimidines [71] and bases like benzylamines, aniline derivates [72] etc. If polar modifiers like water or ethylene glycol are added to the mobile phase, a three- to seven-times higher column efficiency can be achieved owning to a mixed-partition-adsorption mechanism and this also leads to changes in chromatographic selectivity [71].

Diol-bonded packings are highly polar and the strong ability of hydroxyl-groups contained in the ligand (typically a short alkyl chain which may additionally contain an embedded ether linkage) for hydrogen bonding is responsible for its high polarity. Compared to amino-modified silica, diol silica is more stable and a Schiff base formation phenomenon on amino silica between amino groups and reducing sugars (*vide infra*) is eliminated on diol silica [8, 73].

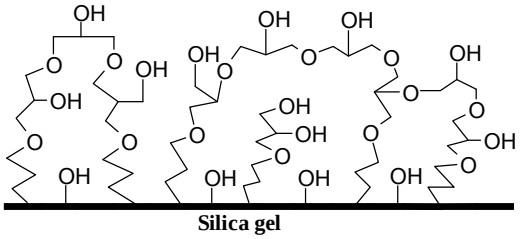


Figure 5. The chemical structure of a diol-type stationary phase (Luna HILIC)

A so-called mixed mode diol-type HILIC column, Acclaim Mixed-Mode HILIC-1, was recently commercialized by Dionex (Sunnyvale, CA, USA). The bonding of this chromatographic material contains both hydrophilic functionalities, *i.e.* diol groups and also hydrophobic functionalities, *i.e.* a C_{10} alkyl chain. This HILIC phase is termed to be a multi modal packing material which means it can be operated either in HILIC mode or in RP mode.

Diol silica columns have been used to separate vitamins, nucleosides, polyols, monosaccharides and ginsenosides [73-76]. Although the high phase polarity of diol-modified silica makes such kind of packings attractive for HILIC separations, a quite limited number of articles yet appeared in literature.

1.4.3.3 Chargeable and permanently charged bonded phases

1.4.3.3.1 Amino-type stationary phase

Amino-modified silica is the first generation of bonded silica to be adopted in HILIC separations (Figure 6) since 1975. Commercial amino silicas are for example Luna Amino (Phenomenex, Torrance, CA, USA) and Zorbax NH_2 (Agilent, Santa Clara, CA, USA).

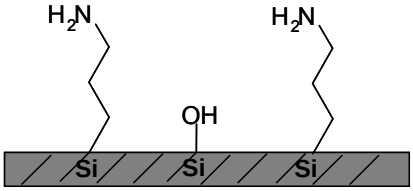


Figure 6. The chemical structure of functional group of amino silica

Bonded amino groups are quite reactive and they can interact with hydroxyl group in sugar molecule forming an amino sugar or can interact with the aldehyd or keton groups in reducing sugars to form Schiff base which causes an irreversible adsorption of analytes on the silica and leads to a deactivation of the column [60]. Probably this is one of the reasons why amino-type silica has not been chosen for carbohydrates separation in recent years. On the other hand, the aminopropyl groups of amino silica are reported to be susceptible to hydrolysis in aqueous eluents which means the aminopropyl ligands will release from the surface of silica and result in a bad reproducibility of data with respect to retention and selectivity and also accompanied by bad peak efficiency in HILIC mode [77, 78] whereas it is depending on the type of amino phase. For example, the aminoquinuclidine-bonded RP/WAX phase that will be introduced later exhibited good chemical stability (*vide infra*) [29]. Furthermore, in HILIC-MS the tendency of the amino groups bleeding of the columnn toward ionization in the positive ion mode bears risk for ion suppression for the analytes [79]. Certainly, the separation characteristics of amino silicas such as retention factor and selectivity are also more or less dependent on the manufactures [31, 80].

Additionally, a significant ion-exchange effect is observed on amino-type columns [22, 31, 81]. This could be induced by the protonated amino groups and potentially deprotonated residual silanols. The pH value of mobile phase has a strongly effect on the separation properties of such chargerable stationary phase. With neutral and acidic mobile phases, the

amine groups in the stationary phase will be positively charged which offers the possibility for an anion-exchange mechanism superimposed on hydrophilic interactions.

Another variant of amino-type stationary phases are organic polymer-based amino phase in which a polyamine is covalently bonded to a copolymer. This type amino column offers extended chemical stability from pH 2-12 and high column efficiency. For example, phenomenex AshaipakNH250D is a polymer-based amino-type stationary phase and it has been utilized for separation of 5-fluorouracil in plasma and tissue in HILIC-APCI-MS mode [82]. This phase exhibits good stability over silica-based amino phases and relatively high efficiency for the analysis of underivatized polar amino acids as well as good MS compatibility in LC-MS [82, 83]. However, polymer-based stationary phases have lower column efficiency compared to silica-based phases.

Amino-bonded stationary phase are frequently utilized for analysis of polar compounds such as carbohydrates, amino acids, sugars, peptides, nucleobases, nucleosides, tetracycline antibiotics etc. in the HILIC elution mode [16, 31, 58, 81, 84-86].

1.4.3.3.2 Anionic stationary phases

A frequently used anionic stationary phase in the HILIC mode is polysulfoethyl A (PolyLC, Columbia, MD, USA). Polysulfoethyl A, poly(2-sulfoethyl)aspartamide silica (Figure 7), is prepared by aminolysis of poly(succinimide) silica with 2-aminoethylsulfonic acid [15]. Polysulfoethyl A is a strong cation-exchange material with a high degree of hydrophilicity. Hence, it is a suitable phase for mixed-mode hydrophilic/ion-exchange chromatography in which hydrophilic interactions overlaid by electrostatic interactions are responsible for the HILIC retention of polar and/or charged compounds. In the HILIC elution mode, even negatively charged solutes can be retained which is beneficial for separation of complex mixtures which contain different solutes with different net charge and/or charge-type [27]. Poly(2-sulfoethyl)aspartamide stationary phases are reported to be prone to column bleeding which is possibly due to a supported hydrolysis of the amide bonds causing a short column lifetime [87].

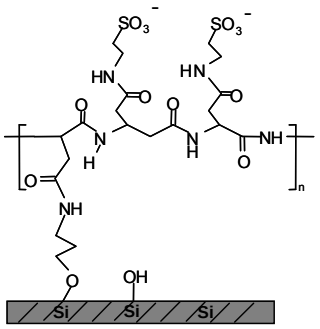


Figure 7. The chemical structure of coating of poly(2-sulfoethyl)aspartamide silica

In the HILIC elution mode, polysulfoethyl A phase has been used for the separations of amino acids, nucleosides, nucleotides, peptides etc. [26, 27, 88].

1.4.3.3.3 Cationic stationary phases

Acclaim mixed-mode WAX-1 (Dionex, Sunnyvale, CA, USA) is a kind of cationic stationary phase and it is a silica-based mixed-mode stationary phase which incorporates both hydrophobic and weak anion-exchange properties. Acclaim mixed-mode WAX-1 is prepared by grafting alkyl amine onto silica (Figure 8a). Additionally, a N-(10-undecenoyl)-3-aminoquinuclidine-modified RP/WAX material (AQ-RP/WAX, figure 8b) was developed by the Lindner group [28]. This phase features distinct interaction sites in one single chromatographic ligand, namely a hydrophobic alkyl chain, embedded polar sulfide and amide groups as well as a terminal bicyclic quinuclidine ring which causes the anion exchange. This multimodal phase can be operated in the RP mode, in the weak anion exchange (WAX) mode and in HILIC mode as well. Accordingly, the AQ-RP/WAX phase shows complementary selectivity to amino-type WAX columns and to conventional C_{18}-RP columns [30]. The AQ-RP/WAX has a slightly higher hydrophobicity for neutral and basic compounds and a slightly weaker anion-exchange for acids than acclaim mixed-mode WAX-1 [30]. This AQ-RP/WAX stationary phase has been reported to exhibit good HILIC capability in analysis of metabolites and peptides etc. [25, 28-30]. Accordingly, this AQ-RP/WAX column has excellent stability and there was no significant loss of performance even after almost 700 runs with urine samples [29].

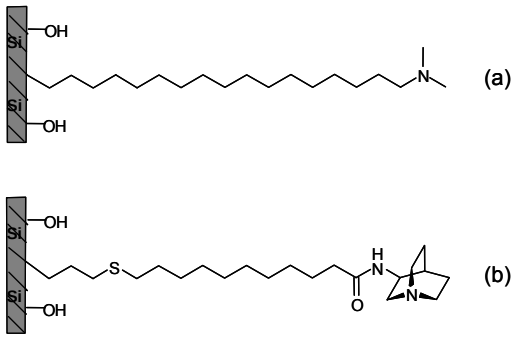


Figure 8. The chemical structure of the coating of the acclaim mixed-mode WAX-1 (a) and AQ-RP/WAX (b)

1.4.3.3.4 Zwitterionic stationary phase

One common zwitterionic stationary phase is a sulfoalkylbetaine-type packing which contains both positive and negative charges in a covalently bonded ligand (Figure 9). The support materials can be polymeric support (commercially called ZIC-pHILIC column) or silica gel (ZIC-HILIC column, both from Merck Sequant, Umea, Sweden) [89-91]. The oppositely charged groups in the sulfoalkylbetaine ligand, *i.e.* strongly acidic sulfonic acid group and strongly basic quaternary ammonium group, are ideally present in 1:1 molar ratio on the same moiety and they are separated via a short alkyl spacer.

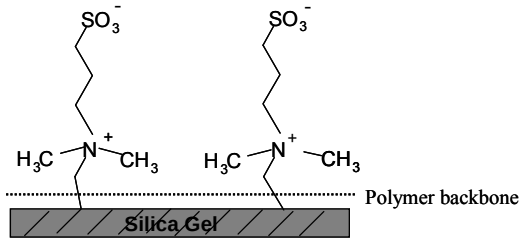


Figure 9. The structure of functional groups of sulfoalkylbetaine-bonded silica (ZIC-HILIC material)

This zwitterionic ligand allows for simultaneous attractive and repulsive electrostatic interactions between the stationary phase and polar charged analytes. But these electrostatic interactions based on sulfoalkylbetaine phase are weaker than those based on normal ion-exchangers and on amino silica as well as on bare silica [92-94]. In theory this sulfoalkylbetaine-type zwitterionic ligand is overall non-charged due to inter-ligand charge compensation effects, but owning to the distal negatively charged sulphonic groups, this sulfoalkylbetaine-type stationary phase is thought to still have a very low net negative

surface charge [95, 96] which causes just very slight strong electrostatic interactions compared to those accessible on amino- and bare silica and it is little affected by the pH value of the mobile phase [22]. In practice, this little pH-effect (in consistent with the polymer coating strategy) offers robustness and a large flexibility in choosing buffer salts for method development [89, 91, 97]. Sulfoalkylbetaine-modified silica has less silanol activity than amide silica and other bonded silica phases [22]. Supposedly, the little residual silanol activity is due to the self-association of the positive charged quaternary ammonium group in the ligand with the residual silanols on the surface which yields an efficient shielding effect [91].

Sulfoalkylbetaine-type zwitterionic ligands have a strong capability to adsorb water molecules from hydro-organic eluents via splitting into hydroxyl anions and hydrogen cations and association with the respective counter-ions in the ligand [91]. This particular feature makes such phases very attractive for HILIC applications and they have been used for separation of salicylic acid derivatives, nucleobases, nucleosides, deoxynucleosides and amino acids [22, 93, 94].

2 Objective

This thesis deals with the development, characterisation, and application of polar modified silica-based stationary phases for HPLC. In particular, the work aimed to further validate the concept of using chromatographic materials containing non-charged polar bondings in the HILIC elution mode. Retention and selectivity characteristics of polar-modified silicas in the HILIC elution mode were investigated according to (i) the type of polar functionalization and (ii) the surface coverage of the ligand. For this purpose the effect of systematic variations of the elution conditions (mobile phase composition, temperature) were studied as well. Overall, this work strived to widen the knowledge on preparation and evaluation of stationary phases combining several selectivity principles as well as to gain deeper insight into the HILIC retention mechanism.

3 Results and discussion

3.1 Development of novel non-charged polar silica packings and their chromatographic evaluation in RP and aqueous HILIC elution modes

Diol–type stationary phases contain hydroxyl functionality on the surface and they are regarded as non-charged phases. In NPLC, diol-bonded silica is alternative phase to bare silica without undesired excessive retention due to the relative weaker hydrogen bonding with the diol layer than with the silanols on the bare silica. Diol columns are also suitable for HILIC separations of highly polar compounds due to the high phase polarity and the good accessibility to hydrogen bonding. Although there are several diol-type columns available on the market and a number of papers have already been published about their practical suitability in HILIC mode [73, 75-77], diol columns are currently not as popular as other HILIC type columns. In this study, we intended to more adequately explore the HILIC potential of diol-type stationary phase. Simultaneously, we wanted to gain an insight into the HILIC retention mechanism by using obtained data on non-charged polar parckings, because the lack of chargeable functionalities on diol silica (apart from the residual surface silanol groups) prevented the strong electrostatic solute-ligand interactions which may potentially affect the actual HILIC retention process. Thus, the delivered information from this study should be useful to understand somewhat better the actual HILIC retention process.

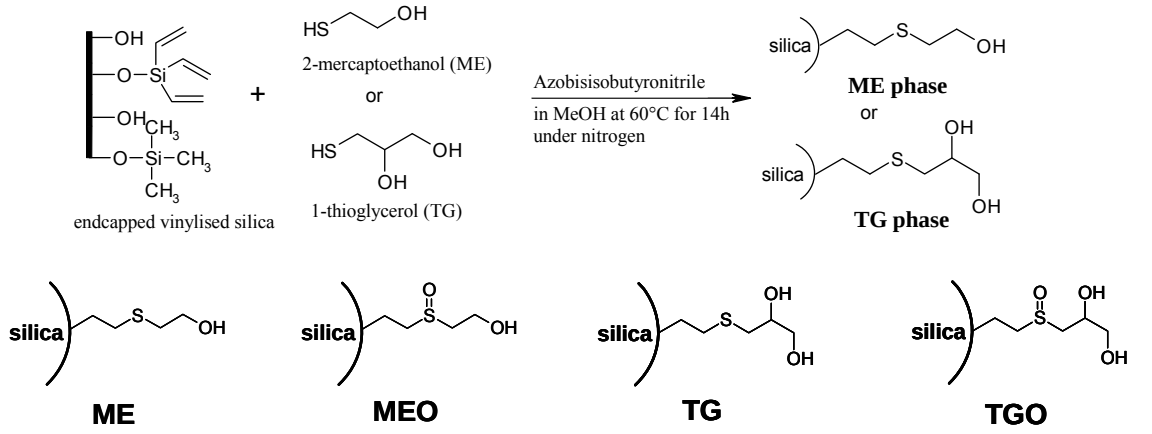


Figure 10. The radical reaction of 2-mercaptoethanol and 1-thioglycerol onto vinylised silica (up) and the proposed structural formulas of all the novel stationary phases (down).

For these purposes, four stationary phases having ligands with hydroxyl groups as well as sulphide and sulfoxide functionalities, respectively, bonded as polar interaction sites were synthesised. So called ME and TG phases were prepared by a radical initiative immobilisation of 2-mercaptoethanol and 1-thioglycerol onto vinylised silica (Figure 10, up). MEO and TGO phases were obtained by an on-phase oxidation of ME and TG materials with excess of hydrogen peroxide in methanol/water solution. The proposed structure formulas are depicted in Figure 10.

The liquid chromatographic characterisations of these packings were examined both in RP mode and HILIC elution mode. Under RP elution conditions, all four developed packings showed a noticeable CH_2-selectivity with respect to butylbenzene and pentylbenzene (α = 1.33 for ME and TG, α = 1.22 for MEO and TGO). The more polar sulfoxide-group delivered higher polarity than the sulphide-group and the hydrophobicity of these four phases increased in the order of TGO < MEO < TG < ME. In the HILIC elution mode, the more polar phase exhibited stronger retention of polar compounds while the selectivity increments owing to phase polarity were different comparing the introduction of additional hydroxyl groups in the chromatographic ligand to sulphur oxidation. The most polar TGO phase delivered satisfying HILIC separation capabilities and selectivities towards nucleosides and water soluble vitamins. The multimodal separation mode of the home-made stationary phases was verified with a set of xanthine derivates which conceptually could be retained in both RP and HILIC mode but showing inversion of elution order depending on the chromatographic mode. For the motive of comparison, all systems were tested with commercial diol-type silica columns under similar conditions. Accordingly, the MEO and TGO phases delivered higher selectivity for cytidine/guanosine (α = 1.3) than ProntoSil diol (α = 1.09) and Acclaim Mixed Mode HILIC-1 (α = 0.88) in the HILIC mode and five water soluble vitamins were baseline separated on TGO phase while some critical pairs were observed on all three commercial columns.

The effects of important experimental parameters, namely nature and amount of organic solvent, salt concentration and pH value of the aqueous buffer as well as the column temperature, on retention and selectivity of selected solutes were in detail studied. The amount of organic solvent had significant effect on retention and polar solutes were stronger retained at higher amount of organic solvent (> 60%, v/v). The investigated buffer pH in a range of 3.0-8.0 acted slightly on the retention of non-charged analytes such as

nucleosides and nucleobases. With increasing concentration of ammonium acetate from 1 to 15 mM a gradual increase in retention of all non-charged solutes were observed in which the gain in retention was stronger for later eluting compounds.

Overall, diol-type stationary phases are very useful for HILIC separations of highly polar compounds. A high phase polarity and polar sites of ligand like the sulfoxide moiety are quite beneficial to generate new retention and selectivity increments. As concerns the still poorly understood HILIC retention mechanisms the obtained data provided valuable preliminary information. It appeared that multiparametric retention mechanism composed of partitioning and adsorptive interactions such as hydrogen bonding between solutes/sorbent surface or solutes/ligand interactive sites were all involved in the generation of retention and selectivity. The phase preparations, the chromatographic evaluation data and more detailed information about the interpretation of HILIC retention process are described in detail in publication (**P1**) (see Addendum #1).

3.2 Comparison of aqueous- and non-aqueous HILIC elution conditions

As has turned out in the initial study (see above), the HILIC retention mechanism seems to be of quite complex nature, at least for the studied solutes and packings. So, in a next step, the role of the polar modifier in the HILIC eluent was investigated. This experimental series was particularly triggered by the fact that HILIC separations are usually carried out using water as the polar protic modifier in acetonitrile-rich mobile phases while the potential of polar-organic eluents (e.g. common in chiral separations) are poorly explored for HILIC-type separation problems. Thus, specific interest lied in the question whether the use of organic polar protic modifiers instead of water are able to deliver different chromatographic selectivities in the separation of some polar compounds. In these quasi polar-organic elution conditions which herein shall be called non-aqueous HILIC (NA-HILIC) various alcohols were investigated, namely methanol, ethanol and 1,2-ethanediol. A comparison of this NA-HILIC mode with the common aqueous eluents (AQ-HILIC) was carried out on the aforemented home-made packings as well as two commercially available diol-type phases and a bare silica column.

The exchange of water by the same percentage of alcohol resulted in a stronger retention of polar compounds (nucleobases, nucleosides). The gain in retention was strongly dependent on the polarity of the protic modifier and it followed mostly in the order of EtOH > MeOH > $Et(OH)_2$ > H_2O. Under isoelutropic conditions sometimes different elution orders were observed compared to AQ-HILIC. This indicated the occurrence of solvent-specific effect on selectivity. Thus, it appears that using non-aqueous protic modifier in the mobile phases is an effective tool for adjusting the retention and selectivity in the HILIC elution mode. However, the various alcohols provided weaker eluotropic strength of the resulting eluents leading to a wider retention time window and the peak shapes of long retained compounds were strongly deteriorated, especially on the bare silica column. The NA-HILIC elution mode with $Et(OH)_2$ and MeOH delivered comparable minimal plate heights to AQ-HILIC elution mode while with EtOH peak efficiencies were mostly worse.

The chromatographic effects of other variables of mobile phase, namely the amount of organic protic modifier, the salt concentration, and the column temperature on retention and selectivity were investigated as well. The retention of solutes was maximal when the amount of the protic modifier was minimal under both AQ-HILIC and NA-HILIC conditions. A significant effect of the eluent salt load was observed in both modes, in AQ-HILIC usually being a gain in retention at higher amounts of salt while in the NA-HILIC mode both negative and positive effects on retention were observed when increasing the salt concentration. Irrespective of the type of polar modifier all solutes exhibited a decrease of retention at higher temperatures while, however, the temperature effects on selectivity were different depending on the type of protic modifier and the solutes.

According to the compiled data, AQ- and NA-HILIC retention processes were both supposed to have adsorptive characteristics and a mixed-mode retention model comprising partitioning and adsorption was responsible for the observed HILIC retentions. This assumption of adsorption phenomenon was supported by some findings: (i) relative retention responded irregularly to a change of column structure (solute structure effect and/or column structure effect), (ii) the effects on retention and selectivity of the introduction of different polar sites to the ligand moiety (sulphoxide- *vs.* hydroxyl-group) were distinctly different which indicated an active participation of chromatographic ligand in the retention process, (iii) a significant inter-column difference was observed for the chromatographic selectivity although the stationary phase (protic modifier layer, according

to partitioning retention model) as well as mobile phase were identical in the separation system on different columns, (iv) distinct solvent effects (polar protic modifier) on selectivity were found. It seems that the adsorptive characteristics in HILIC retention process can be derived from solute-lignad and/or solute-solvent shell interactions. Detailed results of this study can be found in publication (**P2**) (see Addendum#2).

3.3 Comparative chromatographic characterization of RP/WAX columns in RP and HILIC elution modes

The aforementioned interpretations of the HILIC retention process was based on the implementation of non-charged polar packings. However, charged and chargeable packings are also very frequently used in HILIC separations such as ZIC-HILIC, Polysulfoethyl A etc.. In this context we were motivated to increase the understanding of the driving force for HILIC retention on such charged including zwitterions columns. Earlier a set of RP/WAX phases with different RP/WAX ligands were developed in our group (Figure 11). As a characteristic RP/WAX stationary phases they combine a hydrophobic alkyl chain and a ionic functionality in ligand which provide hydrophobic and electrostatic interaction sites in one molecular offering complementary retention mechanisms in one system. Such so-called mixed-mode phases containing distinct chromatographic separation principles are deemed to be attractive for specialized retention and selectivity adjustment. To learn more about the multi-modal properties of these columns and gain an insight into the exact chemistry of the HILIC retention process on such charged packings, these home-made columns were comparatively evaluated together with some commercial packings (WAX-type, RP-type, RP/WAX-type, HILIC-type) in RP and HILIC elution modes.

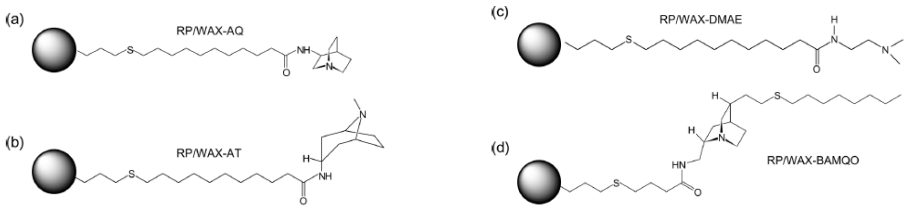


Figure 11. Proposed structural formulas of mixed-mode RP/WAX phases developed in house. (a) N-undecanoyl 3-aminoquinuclidine-bonded; (b) N-undecanoyl 3-aminotropane-bonded, (c) N,N-dimethyl-N-10-undecanoyl-1,2-ethanediamine-bonded and (d) N-butanoyl-(2S, 4S, 5R)-2-aminomethyl-5-[(2-octylthio) ethyl]-quinuclidine-bonded

Under RP elution conditions (i.e. water-rich eluent) the in-house developed RP/WAX columns exhibited sufficient hydrophobicity with $\alpha^{methylene}$ from 1.43 to 1.56 (butylbenzene & pentylbenzene) and anion-exchange capability in terms of retaining the polar acid *o*,*o*-diethylthiophosphate(DETP) (k^{DETP} 12.69-30.75). On a single-mode RP column (Synergi Fusion) the latter solute eluted at the void time of the column. In contrast, on WAX-type phases such as Luna amino CH_2-increment selectivity was not observed while DETP could be well retained. Thus, the RP/WAX materials provided unique retention and selectivity profiles compared to the single-mode stationary phases. Among a set of commercially available RP/WAX columns, the Acclaim mixed-mode WAX-1 showed the most similar retention and selectivity profiles to the home-made RP/WAX-aminoquinuclidine (RP/WAX-AQ) with respect to both hydrophobicity and ion-change capability. Other commercial RP/WAX phases generally showed lower ion-exchange capability (k^{DETP} 0.2-5.8) and they exhibited methylene selectivity in a range of 1.4-1.8.

In the HILIC elution mode, the in-house developed RP/WAX columns and also the commercial Acclaim mixed-mode WAX-1 exhibited good separation capabilities in term of xanthins and nucleosides, while critical peak pairs and/or deteriorated peak shapes were observed on typical HILIC columns such as TSK Gel Amide-80, ZIC-HILIC and Polysulfoethyl A. According to chemometric data treatment by principal component analysis (PCA) of HILIC retention data, the in-house developed RP/WAX columns and Acclaim mixed-mode WAX-1 were of high similarity while the TSK Gel Amide-80, ZIC-HILIC and Polysulfoethyl A columns had high dissimilarity regarding the RP/WAX phases.

Overall, the comparative evaluation of mixed-mode phases demonstrated their applicability in both RP and HILIC elution modes. With regards to HILIC retention process, beside the hydrophilic interactions also electrostatic interactions were observed. For example, a much stronger retention of acids such as ascorbic acid and nicotinic acid was delivered on RP/WAX column than that on TSK Gel Amide-80 which is termed to be a non-charged phase. The hydrophilic interactions were supposed to comprise partitioning and adsorptive interactions (solute-ligand, solute-solvation shell on the surface or solute-residual silanols) on RP/WAX stationary phases. Some similar arguments of the adsorptive characteristics in retention to that of non-charged packings were given on these RP/WAX columns. For example, (i) the log *k* values did not linearly dropp with increasing log D (hydrophilicity

descriptor) in the HILIC mode and compounds with higher number of interactive sites like hydrogen-donor/acceptor in molecular were stronger retained than expected from the log D values; (ii) the correlation of retention factors on RP/WAX-AQ and TSK Gel Amide-80 columns under the same elution conditions was not linear (column-specific effect). According to the obtained data, it is reasonable to conclude that the multi-modal retention mechanisms of RP/WAX phase are based on electrostatic interactions superimposed by hydrophobic and hydrophilic interactions and they explain the distinct behaviour to polar-bonded packings which lack anion exchange sites. More details on the characteristics of RP/WAX phases as well as their unique separation properties compared to RP phases, typical HILIC phases as well as commercially available RP/WAX phases can be found in publication (**P3**) (see Addendum#3).

3.4 Contributions of surface silanols to retention and selectivity properties of bonded silica packings in HILIC

Silanol groups being inevitably present on bonded silica packings may affect retention and selectivity properties not only in RP chromatography (as it is well known from literature) but probably also in HILIC. On polar bonded columns one can imagine that not only the ligand but also the residual surface silanol network is able to develop hydrophilic, i.e. weak electrostatic interactions. Additionally, ionised silanols may also develop strong electrostatic interactions with polar charged solutes (attractive interaction in case of basic solutes, repulsive interactions in case of acidic solutes). Thus, 'silanol activity' for polar bonded columns operated under HILIC conditions is likely of complex mechanistic nature. Literature is devoid of systematic studies that could help answering the question to what extent silanol interactions affect retention/selectivity of polar bonded columns.

In the present work several batches of propylurea-bonded packings (Figure 12) were synthesised which featured different selector loadings, namely 0 (bare silica), 0.38, 0.91, 1.81 and 3.67 $\mu mol.m^{-2}$ ligand surface coverage (calculated based on %N determined by elemental analysis of the bonded phase). The non-charged propylurea-bonded packings excluded extra ionic interactions with ligand, except the desired silanophilic interactions. Hence, the resulting data should be suitable to interpret the actual silanol-activity in the

HILIC elution mode.

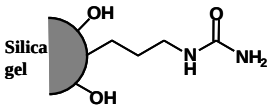


Figure 12. Structural formula of chromatographic ligand

A set of compounds comprising weak/strong acidic, weak/strong basic, zwitterionic as well as non-chargeable solutes was employed for the characterisation of retention properties of the different packings under HILIC elution conditions. The structure formulas of some test compounds are given in Figure 13. Pronounced effects of ligand density on retention and selectivity properties were identified. Most non-charged and acidic compounds experienced a gain in retention from 16% to 286%, when the ligand density was increased from 0 to 3.67 $\mu mol.m^{-2}$ and the retention of acidic compounds was found to be more susceptible to the introduction of the ligand onto the silica surface. The latter finding may be explained by a reduction of (strong) repulsive silanol-solute interaction sites in case of higher ligand density. Zwitterionic solutes and especially bases experienced a loss in retention on more densely bonded phases which is likely due to a reduction of (strong) attractive silanol-solute interactions in case a higher amount of ligand is present on the silica surface which thus blocks in part silanol interactive sites.

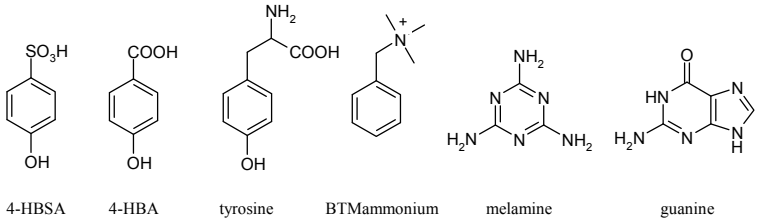


Figure 13. Structural formulas of some test compounds. 4-HBSA: 4-hydroxybenzenesulfonic acid; 4-HBA: 4.hydroxybenzoic acid; BTMammonium: benzyltrimethylammonium.

The effect of salt concentration on retention and selectivity was determined in a range of 3.75 mM to 30 mM ammonium acetate. During an offensive contrast molecular comparison acidic and non-charged compounds showed an increase of retention on both bare silica and bonded packings with increasing salt concentration. For basic solutes the situation was more complex. For example, the weak base melamine showed an increase of retention on all packings at higher salt concentration with eluent ACN/NH_4Ac in water at pH 6.0 (90/10, v/v) (consistent with what is to be expected for a mechanism primarily

driven by ‘‘hydrophilic interactions’’) while the strong base benzyltrimethylammonium consistently displayed mostly a loss in retention at higher salt loads (to be expected in case retention is driven by attractive ‘ionic interactions’). The decrease of retention as observed for some bases at higher salt concentration was stronger on bare silica than on the bonded phases which is consistent with a retention model based on attractive solute-silanol interactions. It can be concluded that using higher salt concentrations in HILIC eluents may not only act on the weak electrostatic and partitioning driven hydrophilic interactions mechanism by increasing retention but it may also influence silanol-solute interactions, e.g. by an ion-pairing like shielding of dissociated silanols with NH_4^+ ions. Thus, the overall ‘salt effect’ may be either an enhancement or a decrease of retention, depending on both the type of analyte and the ligand density as well.

To investigate the role of salt additives more comprehensively, different salts were tested, namely ammonium acetate (NH_4Ac), ammonium formate (NH_4FA), ammonium trifluoroacetate (NH_4F_3Ac) and triethylamine (TEA). Different ammonium salts having the same buffer ${}^{w}_{w}$pH 5 (i.e. pH value adjusted in aqueous phase prior the addition of acetonitrile) will lead to different eluent ${}^{s}_{w}$pH values (i.e. pH value measured in the final eluent). The actual ${}^{s}_{w}$pH value will influence the ionisation states of residual silanol groups as well as solutes. For strong acids, the strongest retention was delivered with NH_4F_3Ac salt (${}^{s}_{w}$pH~5.13) due to a suppression of ionisation of silanol groups. With TEA (${}^{s}_{w}$pH~7.59) and NH_4Ac (${}^{s}_{w}$pH~7.74) salts, lower retentions were delivered for strong acids which may probably be supposed by the higher ${}^{s}_{w}$pH values promoting the ionisation of silanol groups. In contrast, for strong bases the lowest retention was exhibited with NH_4F_3Ac salt due to the suppression of attraction of solutes cations with ionised silanol groups at lower pH and stronger retentions were observed with TEA and NH_4Ac salts. These trends were observed on both bare silica and bonded columns. Compared to strong acids and bases, the retention of neutral compounds was relatively little affected by the nature of salts. It can be seen that the nature of salts and the buffer ${}^{w}_{w}$pH values play a very effective role to retention, especially for charged and chargeable compounds. In other words, effective silanophilic interactions should be considered in the HILIC retention process.

The effects of temperature on retention and selectivity were investigated in the range from 15°C to 55°C. Generally, a decrease in retention was observed for strong acids and non-charged compounds with increasing of column temperature on all columns which is an expected behaviour for retention mechanisms mainly based on hydrophilic interactions. In contrast, the retention of strong bases was increased on all columns. This was ascribed to the prevailing attractive ionic retention mechanism with (residual) silanols for these solutes. Interestingly, weak bases such as melamine showed a decrease of retention at higher temperatures on bare silica and packings with low ligand density while on densely bonded phases for the same analytes a gain in stronger retention at higher column compartment temperature was observed. Thus, it appears that for certain basic solutes the ligand surface coverage particularly impacts the characteristics of the retention mechanism which may shift from an ion exchange driven one at low ligand density to a HILIC-interaction driven one at high ligand density.

Details on this experimental study will be described in a manuscript, which in a preliminary form is obtained as (MS5) in the Addendum#5.

3.5 Application of the developed diol-type packing for liquid chromatographic fingerprint analysis of *Ganoderma spp.* extracts

Ganoderma lucidum (Figure 14) is a valuable medicinal mushroom in Traditional Chinese Medicine (TCM). Wild *G. lucidum* is very rare in the nature and most of mushrooms on the market are artificially cultivated. *G. lucidum* is well known in the folklore due to his medical, therapeutic functions. According to the scientific research, *Ganoderma* contains very rich pharmacological composition and active ingredients including polysaccharides, triterpenes, alkaloids, nucleosides, steroids, proteins, peptides, several amino acids and mineral elements such as zinc, calcium, iron, manganese and so on. The medical efficacy of *G. lucidum* involves cancer, hepatopathy, isomnia, neurasthenia, immune system, anti-ageing etc.. *Ganoderma* comprise more than 200 species and they are mainly distributed in Asia, Australia and Africa. The wide range of *Ganoderma spp.* and the broad geographical origin are calling a reliable and accurate methodology to discriminate *Ganoderma spp.*, differentiate *G. lucidum* samples from different areas and to control their quality.

Figure 14. *Ganoderma lucidum* mushroom.

In this respect, chromatographic fingerprint analysis such as HPLC, gas chromatography (GC) and capillary electrophoresis (CE) experienced considerable popularity due to their powerful separation ability, the comprehensive characterisation of components of samples, and the relative quantification information. Comparatively, HPLC fingerprint analysis has gained the most favour due to the wide range of choices of mobile phases, the repeated use of column, the diverse available detectors, and good reproducibility. It can be simply said a chromatographic fingerprint is a chromatographic pattern of some common, pharmacologically active and chemically characteristic compounds of a kind of real sample like *G. lucidum*. The differentiation of individual samples is accomplished by a comparison of the individual chromatograms to characteristic reference chromatograms.

Chromatographic fingerprint analysis of *G. lucidum* is commonly limited to the use of RP packing due to its developed technical perfection, while RP mode is just suitable for the separation of moderately polar to non-polar compounds. In other words, chromatographic fingerprint information might likely get lost for highly polar compounds. In the present study, chromatographic fingerprint analysis is carried out in both RP mode and HILIC elution mode which is targeting at separations of highly polar compounds. Methodologically, HILIC is highly orthogonal to the RP mode.

Due to the high diversity in the phase chemistry of chromatographic materials applicable in the HILIC elution mode, a set of polar columns, namely a bare silica, the in-house developed TGO silica phase (see Addendum#1) and some commercially available typical HILIC phases like Luna HILIC, Polysulfoethyl A, TSK Gel Amide-80 and ZIC-HILIC were used to investigate stationary phase effects in the framework of fingerprint-based sample discrimination concepts. The motivation for the column comparisons for given samples was the question whether or not the resulting column similarity and dissimilarities are reflected in the abilities to differentiate the *Ganoderma spp*. samples (*G. lucidum, G. atrum, G. sinense, G. tsugae*). The column classification was based on the separation of a small set of compounds including weak/strong bases, weak/strong acids, zwitterions and

non-charged solutes under the same elution conditions to those used for *Ganoderma spp.* extracts. All chromatographic information was recorded by a diode array detector at 254 nm with reference wavelength 450 nm. The chemometric data of separation patterns was delivered by Hierarchical Cluster Analysis (HCA) on retention time using the Number Cruncher Statistical System software. Accordingly, four clusters were delivered by HCA of which bare silica differed most from other stationary phases. TSK gel Amide-80 and Polysulfoethyl A were in one cluster with the most similarity among these columns and ZIC-HILIC had certain similarity to these two columns. Luna HILIC and TGO were separated in another cluster from the other bonded columns.

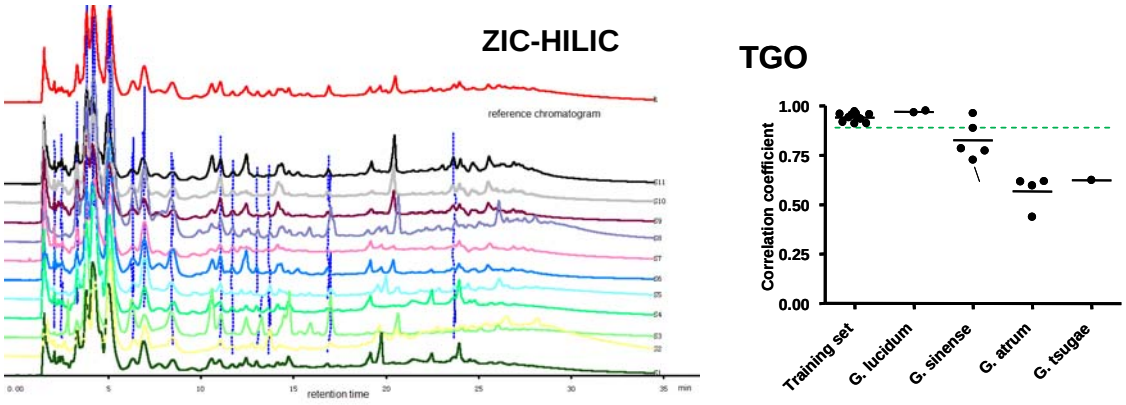


Figure 15. Individual chromatograms of 11 *G. lucidum* specimens (training set) and calculated reference chromatogram as calculated by computer-aided similarity evaluation (left); scatter plots of column-specific correlation coefficients used for species discrimination on TGO column (right)

For sample discrimination from *G. lucidum*, column-specific simulative reference chromatogram of *G. lucidum* was firstly constructed from 11 samples which were collected from some different locations in China. The characteristic reference chromatogram was focused on the retention time windows 0-32 min in HILIC mode and 5-42 min in RP mode in which the most useful information was delivered. One example of the reference chromatogram is demonstrated in Figure 15 (left). The variations of retention times were corrected by the target peak with automatic alignment procedure of a software ‘‘Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine’’ (Version 2004 A). The discrimination of different *Ganoderma spp.* from *G. lucidum* was realised by the similarity of their individual chromatograms to the simulated reference chromatogram of *G. lucidum* and the evaluation of similarity was accomplished by the calculated correlation coefficient of individual sample chromatogram relative to the

characteristic chromatogram. One example for the discrimination of *Ganoderma spp.* on TGO column is given in Figure 15 (right). The lowest correlation coefficient obtained from the *G. lucidum* training set was set as a column-specific cut-off and samples from the validation set having a correlation coefficient to the calculated reference chromatogram below this cut-off were classified as being 'different' from *G. lucidum*. The bare silica column was not able to differentiate *G. lucidum* from other *Ganoderma* species due to a wide range of correlation coefficients obtained from the training set. Successful discrimination of *G.* species from *G. lucidum* could be achieved on all other polar bonded columns. Most samples were correctly classified in the HILIC elution mode. Although the different HILIC columns delivered quite different peak patterns, they were largely similar in terms of their usability for chemometric discrimination of *Ganoderma spp.*. In other words, a change of HILIC columns in was insufficient to avoid sample misclassification.

The discrimination test of *Ganoderma spp.* was also carried out on a Zorbax SB-C_{18} column in RP mode. Although *Ganoderma* sample misclassification was also observed on the C_{18} column, the combining of HILIC and RP data delivered a 100% correct sample discrimination rate for the present sample set. Clearly, such a multi-modal concept of chromatographic fingerprints is advantageous compared to a single-mode approach. The detailed extraction process and the obtained results of this study are given in manuscript (MS4) which is presently under review (see Addendum#4).

The data outlined above were based on UV detection which means the obtained fingerprints are intrinsically limited to compounds with chromophoric groups. However, for the differentiation of such complex biological samples a less selective detector information could be advantageous for obtaining a more comprehensive fingerprint. In other words, by using only UV detection important information may get lost for UV insensitive compounds such as sugars. Thus, in a subsequent study some *Ganoderma* samples were analysed not only using UV detection but also employing evaporative light scattering detection (ELSD). The used *Ganoderma* samples were including 11 *G. lucidum* from different origin, 4 artificial cultivated *G. atrum*, 5 *G. sinense* from different area and one *G tsugae*. The sample extracts were analysed both in HILIC mode (Luna HILIC column) and RP mode (Zorbax SB-C_{18} column) with tandem-detection. Both elution modes were carried out using a binary gradient with an analysis time for 60 min. The chromatographic information was detected at 254 nm using 450 nm as reference

wavelength with UV detector and with the ELSD detector, optimal temperature with regarding to the signal strength was adjusted at 40°C for the *Ganoderma spp.* samples. Comparable sensitivity of two different detectors was observed under the investigated conditions. The combined use of UV and ELSD detection delivered more sample discriminative information compared to the single detection modes. The detailed experimental data and more results will be described in another manuscript (not included in this PhD thesis).

4 Concluding remarks

- The four home-made diol-type columns exhibited multimodal separation properties and are applicable to HILIC and RP chromatography modes. The most polar home-made column TGO delivered high potential for HILIC separations of nucleosides and water soluble vitamins (complementary selectivity to commercial diol columns).
- Phase polarity as well as polar functionalities in the chromatographic ligand like the sulfoxide moiety in TGO and MEO phases is very effective to change HILIC characteristics.
- In HILIC, the impact of silanophilic interactions on retention can not be ignored on polar silica columns and especially for hydrophilic charged analytes they may adopt a prominent role in the retention process.
- The type of protic modifier, as a scarcely explored parameter in HILIC, exhibits an interesting potential to optimise the chromatographic separation for polar solutes
- The amount of organic solvent, the nature and amount of salt and column temperature are all very effective to adjust retention and selectivity of polar compounds.
- In HILIC, complex and multi-parametric retention processes composed of adsorptive (solute-solvent shell on the surface, solute-silanols and/or solute-ligand) and partitioning mechanisms are effective on non-charged polar packings and on charged and chargeable phases the electrostatic interactions are superimposed on hydrophilic interactions.

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6 List of publications and manuscripts

Publication #1 (P1)

Wu J., Bicker W., Lindner W., *Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode*

J. Sep. Sci. 2008, 31, 1492 – 1503.

Publication #1 (P2)

Bicker W., Wu J., Lämmerhofer M., Lindner W., *Hydrophilic interaction chromatography in non-aqueous elution mode: Evaluation of an alternative separation concept for polar analytes on silica-based non-charged polar stationary phases*

J. Sep. Sci. 2008, 31 (16-17), 2971-2987.

Publication #1 (P3)

Lämmerhofer, M., Richter, M., Wu J., Nogueira R., Bicker W., Lindner W. *Mixed-mode ion-exchangers and their comparative chromatographic characterization in reversed-phase and hydrophilic interaction chromatography elution modes*

J. Sep. Sci. 2008, 31 (14), 2572-2588.

Manuscript #4

Chen Y., Bicker W., Wu J., Xie M., Lindner W., *Ganoderma species discrimination by liquid chromatographic fingerprint analysis: A study on column classification and stationary phase effects in hydrophilic interaction chromatography and reduction of sample misclassification rate by additional use of reversed-phase chromatography*

Journal of Chromatography A 2009, -under review-.

Manuscript #5

Wu J., Bicker W., Lindner W., *Effect of surface silanols on retention and selectivity properties of non-charged polar packings in hydrophilic interaction chromatography*

ADDENDUM # 1

Publication (P1)

JunYan Wu
Wolfgang Bicker
Wolfgang Lindner

Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Vienna, Austria

Original Paper

Separation properties of novel and commercial polar stationary phases in hydrophilic interaction and reversed-phase liquid chromatography mode

Four novel nonionic polar stationary phases were synthesised by anchoring first 2-mercaptoethanol and 1-thioglycerol, respectively, onto vinylised silica (ME and TG packings) followed by an on-phase oxidation with excess hydrogen peroxide in aqueous medium which yielded sulphoxide analogues of the embedded sulphide groups, *i.e.* oxidised 2-mercaptoethanol (MEO) and oxidised 1-thioglycerol (TGO) packings. Chromatographic characteristics of these stationary phases were evaluated comparatively to three commercial so-called 'diol' columns. U-shaped response curves of retention factors of adenosine and guanosine with hydro-organic eluents containing 5–95% v/v ACN as well as noticeable CH_2-increment selectivity demonstrated multimodal separation capabilities of the developed amphiphilic materials, *i.e.* columns can be operated both in hydrophilic interaction chromatography (HILIC) and in RP mode. Although the selector ligands were physico-chemically related, considerably differing retention and selectivity patterns were observed in the HILIC mode. Thereby the introduction of additional hydroxyl groups in the chromatographic ligand resulted in selectivity increments that were different from those obtained by sulphur oxidation. For example, a set of five vitamins delivered five different elution orders with the overall seven columns. A close examination of HILIC separations of nucleobases and nucleosides on the developed packings revealed that (i) the amount of ACN in the eluent adopts a pivotal role in adjusting retention, (ii) the linearity of the relationship log (retention factor) *versus* log (volume fraction of water in the eluent) increases with phase polarity in the range of 5–40% v/v water, (iii) the slopes are higher with solutes having more polar interactive sites, (iv) the van't Hoff plots are linear (range 15–45°C) with negative retention enthalpy values ΔH (−4.5 to −14.5 kJ/mol) and (v) the $-\Delta H$ values tend to be higher with more polar phases and more polar analytes. Based on these data the HILIC retention mechanism is described to be composed of both partitioning and adsorption processes. Distinct types of polar interactive sites in the chromatographic ligands may generate mixed-mode HILIC separation conditions that may additionally be superimposed by surface silanol contributions.

Keywords: Diol stationary phases / High-performance liquid chromatography / Hydrophilic interaction chromatography / Mixed-mode separation / Reversed-phase chromatography

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1 Introduction

Among several separation modes in LC such as normal phase, ion exchange or RP, the latter is by far the most frequently used.

This dominant position results from the ability of RP materials and columns thereof to retain and to separate compounds with a large heterogeneity in physico-chemical properties including overall hydrophobicity. However, RP-LC experiences particular limitation for the retention and analyses of very polar compounds like sugars, amino acids or nucleosides. Such solutes typically

Correspondence: Professor Wolfgang Lindner, Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringer Strasse 38, A-1090 Vienna, Austria
E-mail: wolfgang.lindner@univie.ac.at
Fax: +43-1-4277-9523

Abbreviations: HILIC, hydrophilic interaction chromatography; **ME,** 2-mercaptoethanol; **MEO,** oxidised 2-mercaptoethanol; **MeOH,** methanol; **TG,** 1-thioglycerol; **TGO,** oxidised 1-thioglycerol

lack strong interactions with the hydrophobic surface of RP materials and thus elute often nonretained and unresolved from other polar compounds. As far as separation problems with polar target compounds in polar matrix are concerned such an inadequate chromatographic selectivity may cause problems both in analytical (*e.g.* susceptibility to matrix effects in MS-based detection) as well as preparative scale (*e.g.* increased purification efforts). Owing particularly to the growing importance of polar compounds in life science fields such as metabolomics, there is, however, a clear need for chromatographic methods to be able to tackle such separation problems in an adequate manner.

To counter, the limited suitability of RP packings for very hydrophilic solutes, hydrophilic interaction chromatography (HILIC) with polar sorbents and a hydroorganic mobile phase rich in organic modifier, typically ACN, is a valuable alternative chromatographic strategy. Retention and selectivity, respectively, then occurs primarily by hydrophilic interactions and not by hydrophobic forces such as in RP chromatography. Bare silica or silica functionalised with polar entities (amino–, amide–, cyano–, diol– groups, *etc.*) are common packings for HILIC (for review see ref. [1]).

There is a controversy in the literature whether processes of *partitioning* or *adsorption* or combinations thereof are actually responsible for generating retention under HILIC conditions [1]. With respect to diol-type stationary phases, the creation of a selective separation environment for polar analytes under HILIC conditions can be imagined to result mainly from (i) formation of a water-enriched layer on the surface of the sorbent and discriminative partitioning processes between this layer and the mobile phase based on hydrophilicity differences of the analytes and (ii) formation of hydrogen bonds between solutes and the hydroxyl groups of the selector (adsorptive forces).

Several diol-type stationary phases are available on the market and a number of studies document the practical suitability of this approach [2–9]. However, diol columns currently lack frequent use and are less popular compared to other HILIC packings [1]. In this respect, it seems obvious that the HILIC potential of diol-type stationary phases is not yet adequately explored.

The present study was carried out in order to further validate the concept of hydroxyl groups containing selector motifs for HILIC purposes and to widen the knowledge base on preparation and evaluation of stationary phases combining several selectivity principles. In this article, results on the preparation and chromatographic characterisation of four hydroxyl-based silica materials (including diol phases) with additionally embedded functionalities are reported (Fig. 1).

The home-made chromatographic materials were tested both in HILIC and RP mode and the effects of a number of mobile phase parameters on retention characteristics were investigated. For comparison purposes, complementary data were obtained from three commercially available so-called 'diol' phases as well as a representative C_8-RP column.

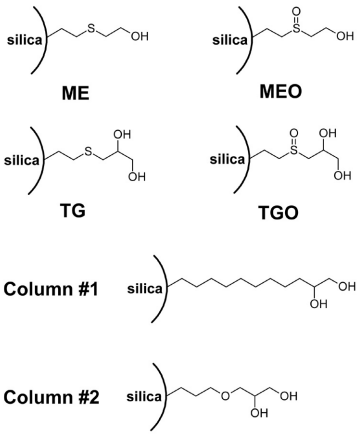

Figure 1. Proposed structural formulas of the home-made and commercial polar stationary phases (structure of column 1 adapted from ref. [10] and that of column 2 taken from personal communication with Dr. S. Lamotte, Bischoff Chromatography, Leonberg, Germany). Note that column 3 seems to have polymeric characteristics with unknown monomeric motifs.

2 Experimental

2.1 Chemicals and materials

HPLC gradient grade ACN and methanol (MeOH) were obtained from VWR International (Vienna, Austria). Water was bi-distilled in-house. Acetic acid, ammonium acetate, aqueous ammonia solution 25%, formic acid, potassium dihydrogenphosphate and potassium hydroxide were of analytical grade (Sigma–Aldrich, Vienna, Austria). 1-Thioglycerol (TG) and 2-mercaptoethanol (ME) were supplied by Fluka (Buchs, Switzerland) and hydrogen peroxide 30% by Merck (Darmstadt, Germany). Daisogel (5 μm, 120 Å, 300 m^2/g) was obtained from Daiso Chemical (Osaka, Japan). An Acclaim Mixed-Mode HILIC-1 column (5 μm, 120 Å, 300 m^2/g, typical carbon content reported to be 8.5% [10], 150 × 4.6 mm id) (column 1) was purchased from Dionex (Vienna, Austria). ProntoSil Diol (5 μm, 120 Å, 300 m^2/g, typical carbon content reported to be 4% [11]) (column 2) as well as a ProntoSil C_8 SH column (5 μm, 120 Å, 300 m^2/g, 150 × 4 mm id, typical carbon content reported to be 10% [11]) originated from Bischoff Chromatography (Leonberg, Germany). A Luna HILIC column (5 μm, 200 Å, 200 m^2/g, 150 × 4.6 mm id, typical carbon content reported to be 5.7% [12]) (column

3) was supplied by Phenomenex (Vienna, Austria). Chromatographic test compounds were purchased from Sigma – Aldrich.

2.2 Preparation of chromatographic materials and column packing

A suspension of vinylised and *via N*-(trimethylsilyl)imidazole (Fluka) end-capped silica (carbon content 8.2%) was reacted with 30 mmol/g ME and TG, respectively, and 0.12 mmol/g α,α-azoisobutyronitrile (Merck) at 60°C for 14 h under nitrogen. The modified silica materials, namely ME and TG stationary phase, were collected by filtration, washed excessively with MeOH and dried at 60°C for 24 h *in vacuo*. Based on the sulphur content determined by elemental analysis selector coverages of 810 μmol/g (2.7 μmol/m^2) and 770 μmol/g (2.6 μmol/m^2) were calculated for ME and TG, respectively. Oxidation of these phases was carried out by treatment with an excess of aqueous hydrogen peroxide solution (37:1 molar ratio with respect to calculated selector load) at 25°C for 5 h. The oxidised silica materials, namely oxidised 2-mercaptoethanol (MEO) and oxidised 1-thioglycerol (TGO), were collected by filtration, washed excessively with water and MeOH and dried at 60°C for 24 h. If the exclusive generation of the sulphoxide analogues of the ligands is assumed (*vide infra*) the calculated selector densities according to elemental analysis (sulphur content) amount to 730 and 700 μmol/g, respectively (2.4 and 2.3 μmol/m^2) for MEO and TGO.

The modified silicas as well as ProntoSil Diol were slurry-packed in 150 × 4 mm id stainless steel columns using MeOH as a carrier solvent.

2.3 Preparation of eluents

Ammonium acetate stock solutions were prepared in concentration levels ranging from 5 to 150 mM in bi-distilled water. pH values were not adjusted except for aliquots of the 100 mM ammonium acetate solution where pH 3.0 was adjusted with formic acid, pH 5.8 with acetic acid and pH 8.0 with aqueous ammonia solution. Mobile phases were obtained by mixing the required buffer stock solution with ACN or MeOH in a range of 5 – 95% v/v. Hydrophobicity and shape selectivity tests were run with MeOH/water mixtures in volume fractions of 60:40 and 70:30, respectively. For the silanol activity test, an eluent composed of MeOH/(20 mM potassium dihydrogenphosphate, pH 7.0 (adjusted with potassium hydroxide)) (65:35 v/v) was used.

2.4 Instrumental set-up

All chromatographic runs were carried out with a 1200 series HPLC system from Agilent (Waldbronn, Germany) equipped with a diode array detector. Chromatograms were acquired and processed with the HP^{3D} Chemstation software. The chosen elution mode, flow rate, column compartment temperature and detection wavelength are noted in the respective figure and table legends. Analytes were injected in concentrations of 0.02 – 0.5 mg/mL in solutions matching the respective chromatographic conditions (injection volume 5 μL). Void times were measured with toluene and ACN/water (90:10 v/v) as eluent. For hydrophobicity and silanol activity characterisations, acetone was used as a void time marker.

3 Results and discussion

In the present study separation characteristics of four novel polar stationary phases were evaluated in the HILIC as well as in the RP elution mode. In order to gain specific insight into the HILIC retention mechanism itself, *i.e.* free from ionic contributions (apart from residual silanol activity), neutral ligands were exclusively investigated. In this respect, one of the main interests resided in how an increase in phase polarity translated into changes in the overall retention and selectivity properties. This was deemed as important preliminary information for follow up designs of stationary phases for specific separation problems and to learn more about the influence of minute selector structure modifications on chromatographic separation capabilities in general.

The first two selector motifs containing a monohydroxy and a dihydroxy (diol) functionality, respectively, together with a sulphide group were immobilised on vinylised silica resulting in the ME and TG stationary phases (see Fig. 1). The presence of thioether functionalities in these structures offered the possibility of introducing additional increments of polarity by an on-phase oxidation with hydrogen peroxide in an aqueous environment leading to the TGO and MEO stationary phases. This mild oxidation route was chosen to avoid oxidation of the hydroxyl functionalities. The oxidised ligand structures are described as sulphoxide analogues of TG and ME, respectively. With a control experiment using butyl sulphide we proved the quantitative oxidation to butyl sulphoxide within the chosen time frame of 5 h. Furthermore, reacting butyl sulphoxide instead of butyl sulphide did not cause noticeable concentration changes of the starting compound (<1%). Therefore, overoxidation to sulphones is not likely to occur under the selected conditions.

The chromatographic data obtained with these four stationary phases were opposed to those delivered by three commercial so-called diol-type HILIC columns as well as a commercial RP column (C_8, not polar embedded). Whereas two of these HILIC columns (for structures see Fig. 1) are marketed as 'pure' diol phases

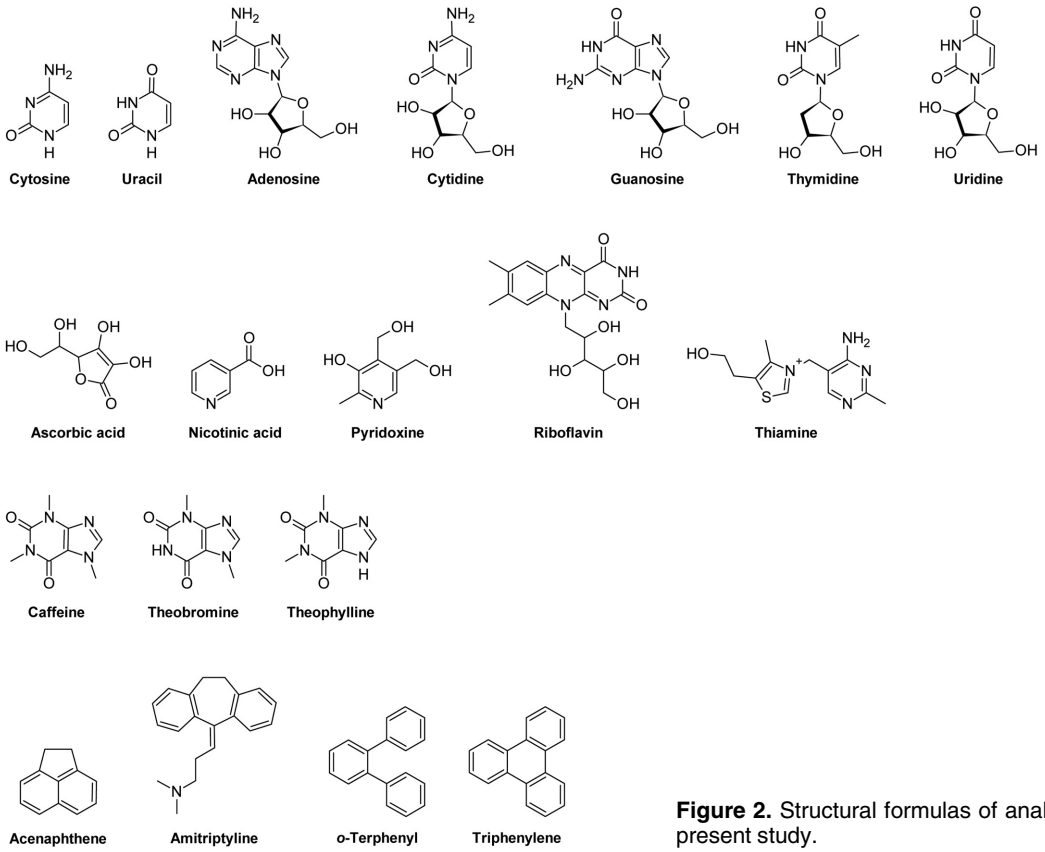

Figure 2. Structural formulas of analytes investigated in the present study.

[11, 12], *i.e.* without explicitly specified hydrophobic properties, one of them is termed to be a multimodal phase featuring both polar (diol) as well as nonpolar (alkyl chain) interactive sites [10]. This concept somewhat resembles our basic idea realised in the synthesised phases, that is combining nonionic polar functionalities with a hydrophobic backbone.

Conceptually, such amphiphilic materials should permit multimodal separations both in the HILIC and RP mode. However, in comparison to our recently developed mixed-mode separation materials of RP/weak anion exchange type [13], which can *inter alia* separate compounds by exploiting simultaneously anion exchange and HILIC [14, 15], one can usually not make use of RP and HILIC interactions at the same time. Nevertheless, due to the different phase polarities and interactive sites we expected unique selectivity properties to arise on the investigated separation materials both in the HILIC and RP mode.

In the following, the chromatographic behaviour of HILIC-typical compounds (nucleobases, nucleosides, vitamins), RP-typical analytes (alkylbenzenes, polyaromatic compounds, amitriptyline) as well as solutes supposed to be retainable both in the RP and HILIC mode (methylxanthines) (Fig. 2) on the in-house developed as well as on the commercial stationary phases is reported.

3.1 HILIC separation capabilities

3.1.1 Nucleoside selectivity

In the first attempt, HILIC properties of the seven separation materials were evaluated for a mixture of five nucleosides, namely adenosine, cytidine, guanosine, thymidine and uridine (Fig. 3).

Comparable trends in elution orders were observed on all seven phases. However, retention factors k and selectivity profiles differed considerably. The ME packing consistently delivered lowest k values which was a clear indication that among the home-made materials ME had lowest polarity. On the other hand, increase in the phase polarity as realised by either introduction of a second hydroxyl group in TG (see Fig. 1) or oxidising the sulphide moieties to sulphoxides (MEO phase) was beneficial to the HILIC-type retention mechanism.

Yet, an increase in retention did not consistently lead to an improvement in chromatographic selectivity α. While for example the α value between cytidine and guanosine was higher on MEO than on ME ($\alpha^{ME} = 1.01$, $\alpha^{MEO} = 1.36$), the opposite figure was found for uridine and adenosine ($\alpha^{ME} = 1.86$, $\alpha^{MEO} = 1.12$) and this trend resembled that observed on the TGO *versus* TG column pair.

Different selectivity patterns were also observed on the commercial HILIC phases. Column 1 was identified to

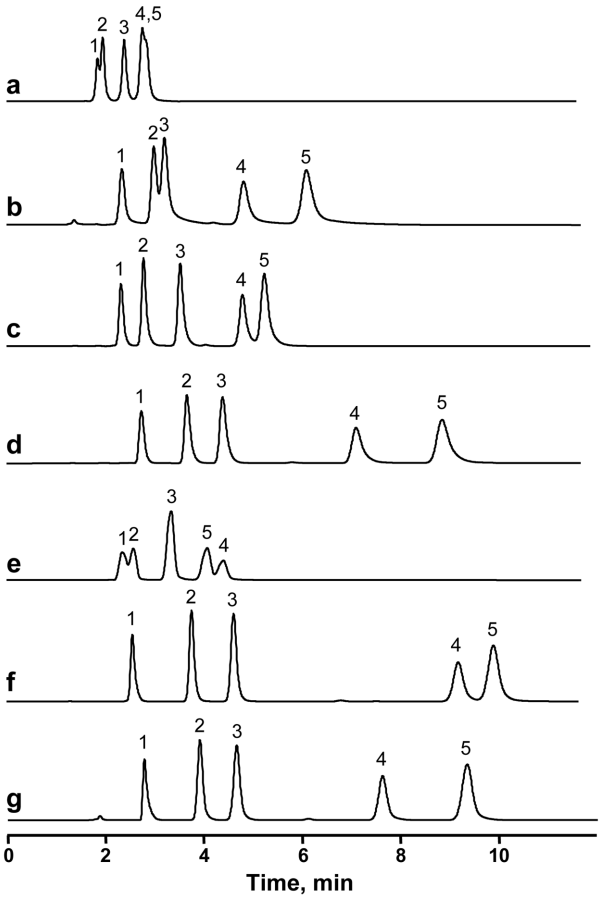

Figure 3. Separation of selected nucleosides in the HILIC mode on various stationary phases, *i.e.* (a) ME, (b) MEO, (c) TG, (d) TGO, (e) column 1, (f) column 2, (g) column 3. Isocratic elution conditions: ACN/150 mM aqueous ammonium acetate solution (90:10 v/v). Flow rate, 1.0 mL/min; temperature, 25°C; detection at 254 nm. Analytes: (1) thymidine, (2) uridine, (3) adenosine, (4) cytidine and (5) guanosine.

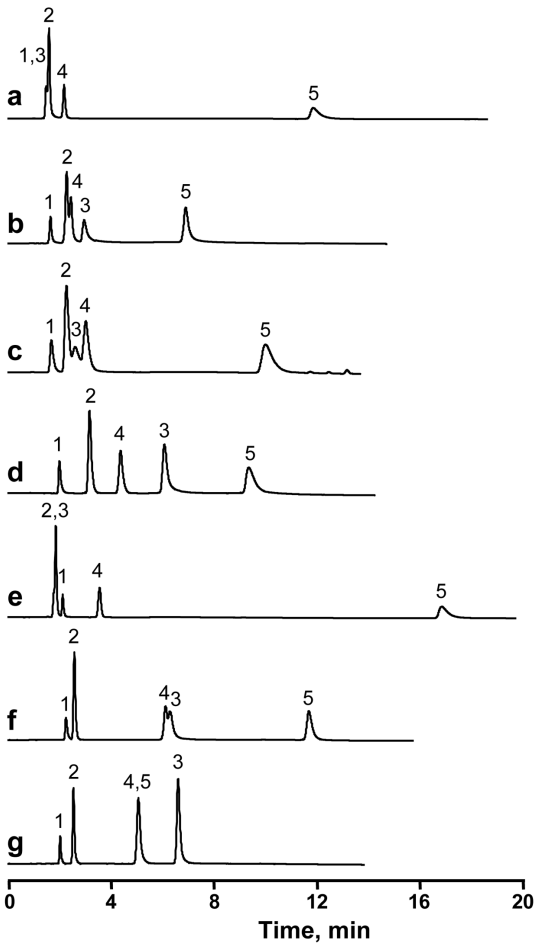

Figure 4. Separation of selected vitamins in the HILIC mode on various stationary phases, *i.e.* (a) ME, (b) MEO, (c) TG, (d) TGO, (e) column 1, (f) column 2, (g) column 3. Elution with ACN–water gradient with fixed amount of 5 mM ammonium acetate, pH 5.8 (acetic acid): 0 min 90% ACN, 3.5 min 90% ACN, 15 min 50% ACN, 20 min 50% ACN, 20.5 min 90% ACN, 35 min 90% ACN. Flow rate, 1.5 mL/min; temperature, 25°C; detection at 270 nm. Analytes: (1) pyridoxine, (2) riboflavin, (3) ascorbic acid, (4) nicotinic acid and (5) thiamine.

possess quite limited HILIC separation capabilities under the chosen conditions (comparable to ME, *e.g.* both could not baseline separate thymidine from uridine in contrast to all other phases), while column 2 and even more column 3 afforded separation profiles which largely matched those obtained with the TGO material.

The slightly better peak performance of the commercial columns as opposed to self-üpacked columns may reside in the fact that for the former the packing procedure was specifically optimised while this was not yet the case for our 'research-only' materials. Column comparisons in present study thus focus exclusively on retention and selectivity profiles and not on peak performance parameters.

3.1.2 Vitamin selectivity

To probe the ability of the diverse mono- and dihydroxy materials in gradient-HILIC mode (descending ACN fraction) a previously reported separation example of five water-soluble vitamins, *viz.* ascorbic acid, nicotinic acid, pyridoxine, riboflavin and thiamine, was adapted [12]. Due to the wide range in pK_a values and hydrophilicity this analyte set was deemed particularly valuable to recognise intercolumn differences.

Indeed, under the chosen conditions the investigated seven columns delivered five different elution patterns (Fig. 4) (note: time program as well as flow rate were not changed for the 4.6 *vs.* the 4.0 mm id columns; absolute retention times can thus not be directly compared). Similar elution orders were found only for MEO, TGO and column 2. On all columns except for column 3 thiamine was eluted latest and except for column 1 pyridoxine showed weakest retention. Considering the quaternary

amine structure of thiamine (Fig. 2) its long retention even under the strongly eluting HILIC conditions (isocratic interval with 50% water) was indicative of the presence of additional silanophilic interactions (note: for a pure silica column this analyte was reported to be eluted only with a more acidic eluent [12]), *cf.* below. Compared to ME, the introduction of sulphoxide groups *via* oxidation of the sulphide moieties (MEO) as well as doubling the hydroxyl functionalities (TG) both helped to increase retention (except for thiamine), but only the phase having both of these features (TGO) allowed baseline separation of all compounds. Interestingly, the elution order of nicotinic acid and ascorbic acid inverted by changing from a nonoxidised to an oxidised phase (ME *vs.* MEO, TG *vs.* TGO) while the elution pattern of all other solutes was affected to a lesser extent. As observed in the nucleoside separation example, the retention pattern of column 1 again resembled largely that observed with the low-polarity ME phase.

The identified intercolumn differences, particularly with regard to the high number of different elution orders, are quite noteworthy considering the fact that all seven packings are basically hydroxyl-containing materials. Thus, other nonionic interactive sites in the ligands such as the embedded sulphide and sulphoxide groups in the home-made materials or residual silanol activity seem to play a relevant role in generating such diverse retention and selectivity patterns.

3.2 Silanophilic interaction at pH 7

Silane-based functionalisation reactions on bare silica cannot address all surface silanols and thus a certain amount will remain available for interactions with analytes and eluent components. This issue is well known from RP chromatography where particularly the retention properties and peak shapes of basic solutes are affected by such silanophilic interactions [16]. Depending on the solute structure and the elution conditions these interactions may be of attractive/repulsive ionic and/or of dipole–dipole (hydrogen bonding) nature. Thus, if HILIC separations are carried out on modified silica packings residual silanol activity should be considered as this may add adsorptive retention increments to the overall HILIC mechanism [1, 17].

As discussed above, the greatly varying retention profile of the quaternary amine thiamine on the investigated stationary phases (Fig. 4) gave hints for silanophilic interactions. To more systematically investigate this aspect, the silanol activity test developed by Neue *et al.* [16] was performed. This test uses 'RP-typical' elution conditions and solutes, *viz.* amitriptyline and acenaphthene, with a MeOH/pH 7 phosphate buffer mobile phase. Although the measured silanophilic interaction refers to this specific mobile phase ('silanophilic interaction at pH 7' [16]) we assume that this trend is valid also for typical HILIC conditions.

Table 1. Retention factor k of amitriptyline and silanophilic interaction value S as calculated according to ref. [16]. Isocratic elution conditions: MeOH/(20 mM KH_2PO_4, pH 7.0 (KOH)) (65:35 v/v). Flow rate, 1.0 mL/min; temperature, 25°C; detection at 254 nm.

Stationary phase	$k^{\text{amitriptyline}}$	S
ME	5.81	3.87
MEO	1.69	3.22
TG	4.08	3.68
TGO	1.94	3.55
Column 1	14.28	4.37
Column 2	1.23	4.52–5.28[a)]
Column 3	0.30	2.08
C_8	16.56	3.64

[a)] Range results from uncertainty in the determination of k value of acenaphthene (k <0.05).

According to the empirically derived equation,

$$S = \ln(k_{\text{amitriptyline}}) - 0.7124 \times (k_{\text{acenaphthene}}) + 1.9748$$

the hydrophobic contribution to the retention of amitriptyline can be largely eliminated and the resulting factor S is a direct measure for silanophilic interactions (if one assumes that under these conditions on present packings other polar interactions do not contribute to the retention to a relevant extent) [16]. Applying this concept to the investigated materials led to S values between 2.1 and 5.3 (Table 1). For both ME and TG higher values were obtained than for MEO and TGO which we ascribe to as a shielding effect of the more polar sulphoxide moiety in the oxidised packings compared to the sulphide group in ME and TG. Both columns 1 and 2 (to our knowledge not end-capped materials) resulted in higher S values than the in-house end-capped materials whereas with column 3 (selector supposed to be of polymeric nature) the lowest S value within the whole set of packings was yielded.

The extent of silanophilic interactions measured with this test under RP conditions actually resembles well the retention trend of thiamine under gradient HILIC conditions (Fig. 4), *i.e.* columns with high S values tend to retain thiamine more strongly. Thus, the retention of thiamine under the conditions specified in the caption of Fig. 4 is described to be the result of partitioning and/or ligand-related adsorptive hydrophilic interactions and additional (ionic) interactions with residual silanols. This finding illustrates that in the case of separating charged solutes in the HILIC mode even on a neutral bonded silica-based packing surface silanol-related contributions need to be considered. Unlike RP chromatography, in the HILIC mode the availability of silanophilic interactions is not undesired *per se* – quite the opposite, if carefully controlled by the mobile phase conditions it

Table 2. Absolute hydrophobicity ($k^{\text{pentylbenzene}}$) and CH_2-increment selectivity ($\alpha^{\text{pentylbenzene/butylbenzene}}$) of synthesised and commercial stationary phases. Isocratic elution conditions: MeOH/water (60:40 v/v). Flow rate, 0.8 mL/min; temperature, 25°C; detection at 254 nm.

Stationary phase	$k^{\text{pentylbenzene}}$	$\alpha^{\text{pentylbenzene/butylbenzene}}$
ME	1.09	1.33
MEO	0.35	1.22
TG	0.93	1.33
TGO	0.24	1.22
Column 1	3.40	1.54
Column 2	0.05	1.00
Column 3	0.05	1.00
C_8	20.62	1.74

adds to the tools available for adjusting retention and selectivity.

3.3 RP separation capabilities

As discussed earlier, the newly synthesised HILIC phases were supposed to also possess a certain degree of hydrophobicity. It was beyond present work to characterise comprehensively RP properties, however, basic experiments were carried out to get an idea about absolute hydrophobicity, hydrophobic selectivity and shape selectivity.

Absolute hydrophobicity was determined following the concept of Tanaka's test by determination of the *k* value of pentylbenzene [18]. Accordingly, the hydrophobic separation capability in terms of methylene selectivity was deduced from the α value of the solute pair butylbenzene/pentylbenzene. Elution with a MeOH fraction of 60 instead of 80% v/v as given by the original protocol was required due to the rather low retention of these solutes on most of the discussed columns (Table 2).

Among the developed packings ME and TG delivered identical α values of 1.33, whereby the absolute retention of pentylbenzene on TG was reduced by about 15% compared to ME. A more pronounced decrease in selectivity and retention was observed after sulphur-oxidation. On MEO and TGO, the CH_2-increment α value was 1.22 each and *k* of pentylbenzene was reduced by approximately two-third compared to the respective nonoxidised analogue. Thus, in terms of absolute hydrophobicity the developed materials follow the order TGO < MEO < TG < ME.

Drastic differences in hydrophobicity were observed on the commercial materials. Both columns 2 and 3 were not capable of separating the test solutes and they eluted virtually nonretained. On the other hand, column 1 possessed considerable CH_2-increment selectivity (α = 1.54), which approximated the reference value of the selected C_8 phase (α = 1.74) to the greatest extent. However, with regard to absolute hydrophobicity the RP phase delivered 6-fold higher *k* values compared to column 1 and for example almost 19-fold higher values compared to ME.

Separation of the shape selectivity test mixture *o*-terphenyl (twisted out of plane) and triphenylene (planar) [19] on the developed phases gave indications that sulphur-aromatic interactions provide a noticable effect on separating π-aromatic solutes of different sterical requirements (eluent MeOH/water 70:30 v/v). In particular, oxidising the sulphide moieties (ME to MEO, TG to TGO) caused a substantial increase in the α value ($\alpha^{ME} = 1.74$, $k^{o\text{-terphenyl}} = 0.70$; $\alpha^{MEO} = 2.55$, $k^{o\text{-terphenyl}} = 0.41$; $\alpha^{TG} = 1.85$, $k^{o\text{-terphenyl}} = 0.62$; $\alpha^{TGO} = 2.74$, $k^{o\text{-terphenyl}} = 0.33$), which is in agreement with data we observed previously on sulphur-containing RP materials [20, 21]. Along this line, we may argue that oxoaromatic interactions also generate shape selectivity to a certain extent. However, one has to be aware that the meaning of 'shape selectivity' itself is not yet adequately explored for such short-chain ligands as well as for polar selectors in general. Therefore, further studies are required until sound interpretations can be made.

3.4 Multimodal separation capabilities

Based on the aforementioned characterisation studies of the newly developed stationary phases it was found that they possess abilities for both hydrophilic and hydrophobic molecular distinction. Although conceptually different the accessibility of both HILIC and RP separation modes with a single column can be of advantage.

Caffeine, theobromine and theophylline all feature a purine structural element and despite their methylated sites still retain a certain degree of polarity (Fig. 2). This makes them suitable for a HILIC-based separation, though commonly RP chromatography is performed for these solutes [22].

Operating the investigated columns in the HILIC mode (95% ACN) caused these three methylxanthines to elute in order of increasing hydrophilicity, *i.e.* caffeine showed weakest retention on all columns (Fig. 5). The structural isomers theobromine and theophylline turned out to be the critical peak pair under these conditions and eluted unresolved from each of the tested columns.

Opposed to that, in the RP mode (5% ACN) a successful simultaneous separation of all three compounds could be accomplished with the ME packing and column 1, respectively (most hydrophobic phases, see Table 2). Here elution order was inverted, *i.e.* it became a function of hydrophobicity. Doubling the amount of hydroxyl groups in TG *versus* ME did not change the respective *k* and α values to a significant extent while, on the other hand, introduction of sulphoxide moieties (ME *vs.* MEO, TG *vs.* TGO) caused all solutes to elute considerably earlier.

This example confirmed the true multimodal character of the home-made chromatographic materials and

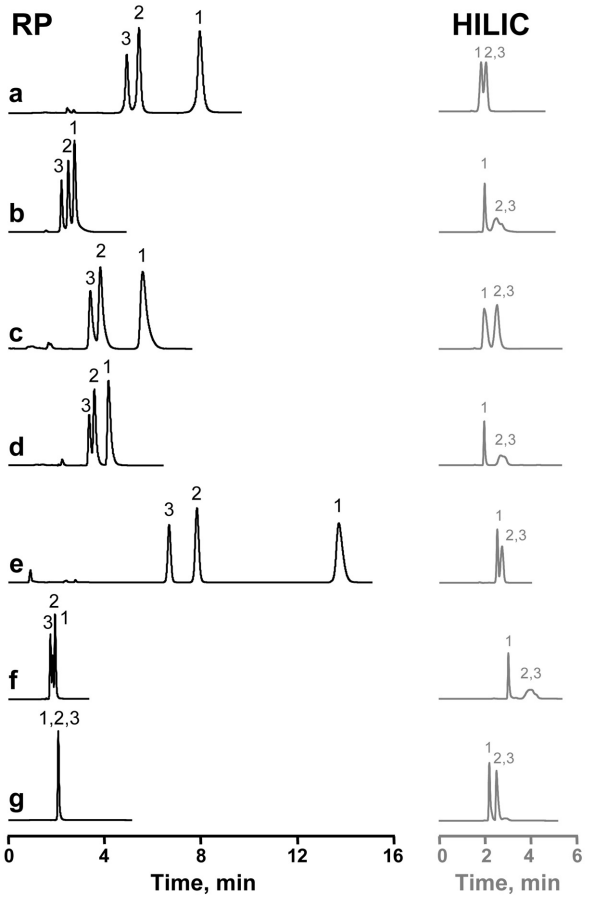

Figure 5. Separation of selected methylxanthines in the RP mode (black lines) and HILIC mode (grey lines) on various stationary phases, *i.e.* (a) ME, (b) MEO, (c) TG, (d) TGO, (e) column 1, (f) column 2, (g) column 3. RP isocratic elution conditions: ACN/5 mM aqueous ammonium acetate solution (5:95 v/v). HILIC isocratic elution conditions: ACN/100 mM aqueous ammonium acetate solution (95:5 v/v). Flow rate, 1.0 mL/min; temperature, 25°C; detection at 270 nm. Analytes: (1) caffeine, (2) theophylline and (3) theobromine.

demonstrated how the orthogonal switch from HILIC to RP mode may be successfully used to solve a given separation problem.

3.5 Impact of mobile phase variables on retention and selectivity

A few experimental parameters were selected to study in more detail their influence on retention and selectivity of selected nucleobases and nucleosides, namely nature and amount of organic modifier, salt concentration and pH of the aqueous eluent fraction and column compartment temperature. Thereby, valuable information on the mechanisms responsible for generating retention in the HILIC mode could be derived.

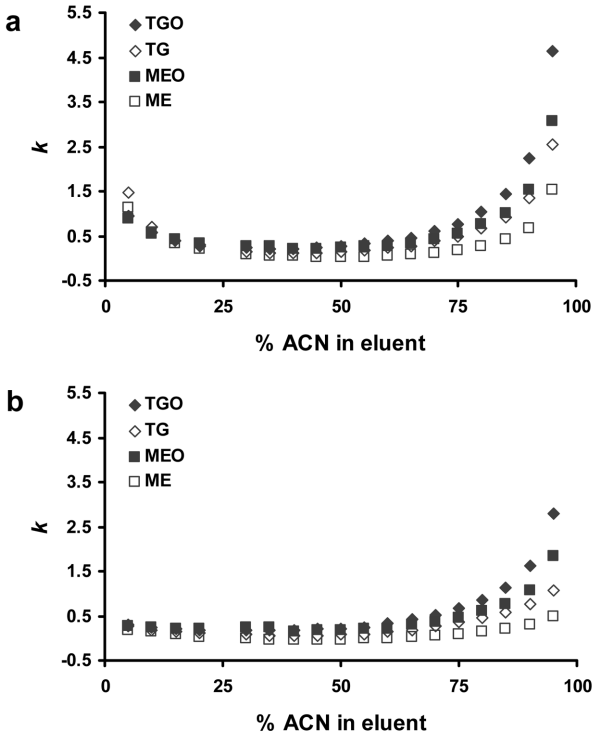

Figure 6. Plots of *k versus* % ACN of (a) adenosine and (b) uridine obtained with the four novel home-made materials. Elution in isocratic mode with ACN/water mixtures of varying ACN fractions (5–95% v/v) and a constant amount of 1 mM ammonium acetate. Flow rate, 1.0 mL/min; temperature, 25°C; detection at 248 nm.

3.5.1 Nature and amount of organic modifier

The necessity of ACN in generating an environment for attractive and discriminative interactions of the solutes with the polar nonionic stationary phases at high organic modifier fractions was proven by a control experiment in which the protic MeOH was used instead of nonprotic ACN. For example, on TGO almost no retention and selectivity could be obtained for the peak pair uracil/uridine (k of both 0.31) with an eluent composed of MeOH/20 mM aqueous ammonium acetate (95:5 v/v) while switching to the corresponding ACN system led to $\alpha = 2.07$ ($k^{\text{uracil}} = 1.36$, uracil eluted before uridine).

The effect of the ACN fraction in the hydro-organic eluent was studied in a range of 5–95% v/v. Figure 6 exemplifies the findings for adenosine and uridine. Both solutes experienced markedly increased retention at higher amounts of ACN (>60%). This is in line with the perception of a HILIC-type retention mechanism. On the other hand, at low fractions of ACN (<20%) the emergence of a significant RP-like retention was realised for the purine base adenosine (and to a lesser extent also for guanosine), but not for the pyrimidine bases uridine and cytidine as well as the nucleobases cytosine and uracil. This effect

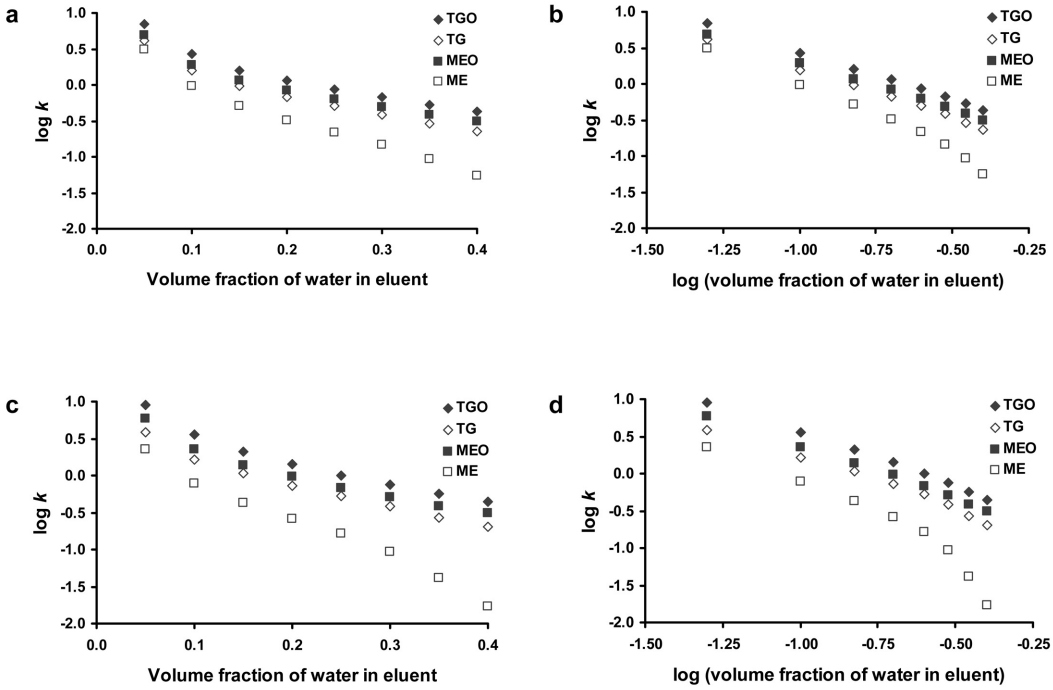

Figure 7. Plots of log *k versus* volume fraction of water in the eluent of (a) cytosine and (c) cytidine as well as plots of log *k versus* log (volume fraction of water in the eluent) of (b) cytosine and (d) cytidine as observed on the four novel HILIC-type materials. Experimental conditions are given in figure caption of Fig. 6.

was – as expected – most pronounced with the nonoxidised and thus less polar phases. In accordance with the methylxanthines separation discussed above, this documents multimodal properties of the developed stationary phases.

As suggested by Hemström and Irgum [1] and following the fundamental relationships between *k* and elution strength established for partitioning in RP chromatography and adsorption in normal-phase chromatography, a gross picture on the prevailing HILIC retention mechanism can be obtained by plotting both log *k versus* the volume fraction of water in the eluent and log *k versus* log (volume fraction of water in the eluent). Linearity in lin–log plots can thereby be taken as an indication for a partitioning process and linearity in log–log plots as an indication for an adsorptive retention mechanism.

As illustrated in Fig. 7 for cytosine and cytidine considerable intercolumn differences occurred in the plotted range of 5–40% v/v water in the eluent (similar trends were observed for all other studied nucleobases/nucleosides). It was eye-catching that for ME the lin–log plot was more suitable to establish a linear dependence while with the more polar phases only the log–log model delivered adequately linear relationships. For example, linear regression analysis of the log–log plots provided r^2 values for MEO and TGO between 0.996 and 0.999.

On the one hand, the nonlinear behaviour of ME in the log–log but not in the lin–log plots suggests that under the specified elution conditions and with the selected solutes partitioning is the prevailing mechanism for this packing. Transition to a curvature in the lin–log plots at low water content (and correspondingly to a linear behaviour in this range in the corresponding log–log plots) indicates, however, that with high ACN fractions adsorptive interactions also come into play [1]. In contrast to ME, such a clear trend could not be obtained for TG.

On the other hand, with an increase in phase polarity by sulphur oxidation adsorptive interactions seem to become more relevant or even dominant for retention (reflected in the linear relationships of MEO and TGO in the log–log plots). Furthermore, by analysing the same set of solutes under similar elution conditions on column 2 it could be demonstrated that also on this sulphoxide-free packing retention has adsorptive characteristics (linearity in log–log plots with r^2 values >0.996).

The highest slope value in the log–log plots was obtained for the more polar cytidine and the most polar TGO packing (TGO: slope cytidine −1.44, slope cytosine −1.31; MEO: slope cytidine −1.39, slope cytosine −1.30; column 2: slope cytidine −1.37, slope cytosine −1.31). In accordance with Hemström and Irgum [1], this finding can also be taken as a viable indication for the presence of adsorptive interactions, *i.e.* solutes having a higher number of polar sites, that is here the ribose structural element in cytidine but not cytosine, interact with polar

interaction sites offered by the chromatographic ligand to a greater extent (and *vice versa*).

Although a complete transition from a partitioning-dominated to an adsorption-driven chromatography upon oxidation of embedded sulphide groups is not likely, these preliminary data demonstrate clearly the distinct impact of the chemical nature of polar functionalities in a chromatographic ligand on retention in the HILIC mode. Based on that it is justified to propose a kind of 'mixed-mode' retention mechanism for the developed packings which is composed of adsorptive interactions and partitioning processes. Complementary supportive data for this hypothesis come for example from the presented differential changes in nucleoside selectivity comparing oxidised with nonoxidised phases (Fig. 3) as these observations cannot be satisfactorily explained with a pure partitioning process which is commonly invoked to describe retention in the HILIC mode.

Nevertheless, one should bear in mind that for the developed packings considerable silanol activities were determined (*vide supra*) which potentially cause additional adsorptive contributions to retention. The latter may not necessarily be of ionic nature and may thus become relevant also for compounds being neutral or being noncharged at the chosen elution conditions.

3.5.2 Salt concentration and pH of buffer

The amount of ammonium acetate in the eluent also had an effect on retention and selectivity of the selected nucleobases and nucleosides. Upon increase in the salt concentration in the final hydro-organic mixtures from 1 to 15 mM (90% ACN) retention for all solutes increased in a gradual fashion whereby later eluting compounds were in direction more affected. Further studies are underway to investigate the impact of salt concentration on retention and selectivity in the HILIC mode is more detail. As, however, expected, a variation in the eluent pH (investigated in the range of pH 3.0 – 8.0) did not generate retention and/or selectivity shifts to a relevant extent (*e.g.* retention varied less than 10% for both nucleobases and nucleosides on all home-made materials).

Thus, in contrast to the nature and amount of organic modifier both the investigated ranges of salt concentration and eluent pH were found to impact retention of the selected nucleobases and nucleosides to a minor extent on the developed packings. This indicates considerable stability of the elution conditions for these stationary phases and solutes which is in line with the general observations for HILIC-type diol phases but – as reported earlier – particularly with regards to the pH stands in sharp contrast to HILIC materials featuring ionic selector motifs [23, 24].

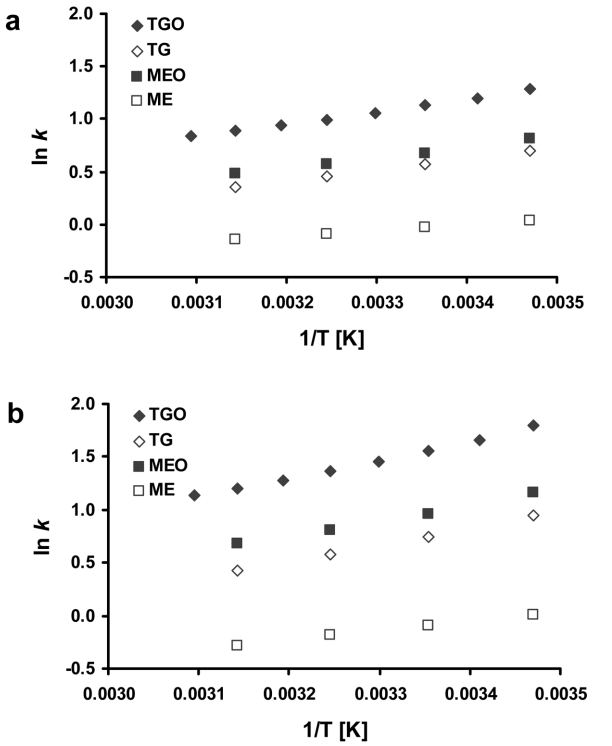

Figure 8. Van't Hoff plots ln *k versus* 1/*T* (K) of (a) cytosine and (b) cytidine as observed on the four novel HILIC-type materials. Isocratic elution conditions: ACN/100 mM aqueous ammonium acetate solution (90:10 v/v). Flow rate, 1.0 mL/min; temperature range, 15–45°C (TGO: 15–50°C); detection at 248 nm.

3.5.3 Column temperature

As a further parameter the effect of the column compartment temperature was investigated in the range of 15 – 45°C (15 – 50°C for TGO). The obtained ln *k versus* 1/*T* plots (van't Hoff plots) showed a linear relationship for all of the developed phases as exemplified in Fig. 8 for cytosine and cytidine. Correlation coefficients r^2 were except for uracil (range 0.91 – 0.99) consistently better than 0.99.

The investigated temperature range was narrow and nonlinearity over a wider temperature range cannot be completely ruled out. However, the observed data strongly point towards the validity of the van't Hoff equation

$$\ln k = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} + \ln \Phi$$

where ΔH and ΔS are enthalpy and entropy changes for the retention interaction (J/mol), R the gas constant ($J \cdot mol^{-1} \cdot K^{-1}$), T the temperature (K) and Φ is the phase ratio.

Calculated ΔH for the home-made materials adopted values between −4.5 and −14.5 kJ/mol which is comparable to the values obtained with column 2 (−8.6 to

Table 3. Retention enthalpies ΔH (kJ/mol) of selected nucleobases and nucleosides as obtained from van't Hoff plots. For experimental conditions see Fig. 8.

Stationary phase	ΔH (kJ/mol)					
	Uracil	Cytosine	Adenosine	Uridine	Guanosine	Cytidine
ME	−6.4	−4.5	−7.6	−9.9	−7.2	−7.4
MEO	−7.4	−8.3	−10.5	−11.0	−11.1	−12.1
TG	−5.2	−8.8	−11.5	−12.1	−13.2	−13.3
TGO	−4.5	−9.7	−11.1	−11.7	−12.6	−14.5
Column 2	−8.8	−8.6	−10.0	−13.6	−13.3	−14.5

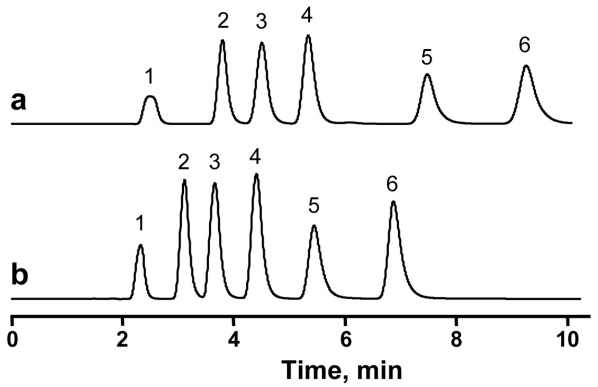

Figure 9. Separation of (1) uracil, (2) uridine, (3) adenosine, (4) cytosine, (5) cytidine and (6) guanosine on TGO at (a) 25°C and (b) 50°C. Other experimental conditions are given in figure caption of Fig. 8.

−14.5 kJ/mol) (Table 3). Earlier data on the temperature-dependent retention of cytosine on four different HILIC packings, *viz*. amino phase, amide phase, zwitterionic phase, bare silica, under similar elution conditions delivered ΔH values between −4.6 and −7.5 kJ/mol [24]. These figures agree reasonably well with those found for this solute on the herein presented new materials and column 2 (−4.5 to −9.7 kJ/mol).

Except for uracil the low-polar ME phase delivered lowest retention enthalpies whereas for the other packings no clear dependency on phase polarity could be identified. Yet, it was obvious that the selected nucleosides gave consistently higher $-\Delta H$ values than the selected nucleobases (*e.g.* $\Delta H_{\text{cytosine}}$ (TGO) = −9.7 kJ/mol, $\Delta H_{\text{cytidine}}$ (TGO) = −14.5 kJ/mol) which in other words means that in this experimental set-up a change in temperature affects retention of nucleosides more strongly than that of nucleobases (Fig. 9).

As proposed above, additional polar functionalities in solutes may allow for additional adsorptive interactions with polar ligand motifs. According to this, the higher retention enthalpies of nucleosides compared to their corresponding nucleobases indicate stronger specific binding *via* these mechanisms, also corroborated by the fact that ME provided these interactions to a lesser extent than the more polar phases. Although the possibility of adsorptive silanophilic interactions with solutes and thus their influence on the measured thermodynamic data still has to be taken into account in our opinion, this interpretation is consistent with the above discussed retention dependency of nucleobases and nucleosides on the water fraction in the eluent and thus furthers the interpretation of a mixed-mode retention mechanism which is composed of partitioning and adsorption.

4 Concluding remarks

In the present study, multimodal separation properties of four home-made nonionic packings with different phase polarities, each containing hydroxyl groups and sulphide- or sulphoxide-functionalities as ligand motifs were investigated in comparison to three commercial so-called diol columns. For some of these chromatographic materials, true multimodality was demonstrated, *i.e.* retention and selectivity could be obtained both in the HILIC and RP mode.

Literature is yet devoid of conclusive experimental evidence on how exactly retention is generated on nonionic polar packings in the HILIC mode. In this respect, we strived to demonstrate that retention and selectivity profiles of polar packings containing structurally quite related ligands that are being tested under similar elution conditions may vary considerably. In particular it could be shown how effective a differential introduction of phase polarity, either by converting embedded sulphide groups to sulphoxides and/or increasing the amount of hydroxyl groups on the chromatographic ligand changes HILIC characteristics. Each of these functionalities may add specific retention and selectivity increments and thus the presence of different nonionic polar motifs in a single ligand may lead to quasi-'mixed-mode HILIC' (note that this is conceptually different from mixed-modal separations with ionic sorbents and charged solutes based on the combination of hydrophilic interactions and ion exchange). This is regarded as an attractive and promising way to solve tricky separation problems with polar compounds.

Our results suggest that depending on the availability and the type of polar interactive sites in both the packing and the solute a multiparametric retention process composed of partitioning of the compounds between a water-enriched sorbent surface layer and the less polar eluent as well as adsorption *via* weak interactions such as hydrogen bonding and other dipole–dipole forces is responsible for retention and selectivity. Such an intermediate HILIC retention model was suggested earlier to explain separations of oligosaccharides on CD-based stationary phases [25] and was also a main outcome of a recent literature study which included a thorough re-evaluation of

earlier HILIC data [1]. However, it is noted that when working with modified silica-based HILIC packings a particular caveat resides in interpreting retention data without considering residual silanol activity also. Depending on elution conditions and solute structure the latter may potentially affect retention *via* ionic and/or nonionic interactions thereby resulting in HILIC separations 'contaminated' with silanophilic contributions.

Valuable attempts were recently carried out to predict retention of analytes on HILIC phases of diol- and poly(vinyl alcohol) type [3, 26] based on computational methods. However, not least because of the lack of standardised HILIC phase characterisation and selectivity tests [27] to date the development of HILIC-type methods is still largely driven by trial-and-error and remains to be a phenomenological approach. Nevertheless, the preparation and evaluation of new HILIC-type chromatographic materials is a step forward to widen our understanding about retention mechanisms thus helping to improve the molecular distinction capacity of polar packings in a dedicated manner and to extract the most influential variables to adjust optimal chromatographic conditions.

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ADDENDUM # 2

Publication (P2)

Wolfgang Bicker
JunYan Wu
Michael Lämmerhofer
Wolfgang Lindner**

Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Vienna, Austria

Original Paper

Hydrophilic interaction chromatography in nonaqueous elution mode for separation of hydrophilic analytes on silica-based packings with noncharged polar bondings*

Chromatographic effects of dedicated stationary and mobile phase variations in hydrophilic interaction chromatography (HILIC) were investigated using a set of nucleobases, nucleosides and deoxynucleosides as polar test solutes. Retention and selectivity profiles were comparatively mapped on four in-house developed silica materials modified with short alkyl chains (C_4, C_5) which carry hydroxyl functionalities (including diol motifs) as well as embedded sulphide or sulphoxide groups. These data were complemented by results obtained with two commercially available diol-type phases and a bare silica column. Besides elucidation of packing-related aspects this work concentrated specifically on extending aqueous HILIC (AQ-HILIC) to nonaqueous polar-organic elution conditions herein termed NA-HILIC. The exchange of the polar modifier water by various alcohols in ACN-rich mobile phases containing 5 mM ammonium acetate decreased the eluotropic strength of the resulting eluents. The gain in retention largely followed the order ethanol (EtOH) > methanol (MeOH) > 1,2-ethanediol ($Et(OH)_2$) and was accompanied by distinct effects on chromatographic selectivity. For example, on the most polar home-made packing the purine nucleoside selectivity guanosine/adenosine increased from 2.25 in the AQ-HILIC ($k^{guanosine}$ = 8.3) to 7.33 ($k^{guanosine}$ = 59) in the NA-HILIC mode when EtOH was employed as NA modifier while this value was 5.84 and 2.93 with MeOH and $Et(OH)_2$, respectively (eluent: 5 mM ammonium acetate in ACN/modifier 90:10 v/v). Besides the type of protic modifier its percentage as well the retention and selectivity effects upon varying the ammonium acetate concentration and column temperature, respectively, were also investigated. Notable inter-column differences were found for all of these elution parameters. A mixed-mode retention model composed of partitioning and adsorption is proposed for both AQ- and NA-HILIC retention processes. The potential of (i) the implementation of novel polar bondings (such as ones containing sulphoxide functionalities) and (ii) the comprehensive exploitation of elution variables (type of protic modifiers, salt, *etc*.) for providing new selectivity increments to the separation of polar analytes in HILIC is emphasised.

Keywords: Diol stationary phases / High-performance liquid chromatography / Hydrophilic interaction chromatography / Nonaqueous chromatography / Polar-organic elution mode

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1 Introduction

The analysis of polar compounds is of importance to many branches of life sciences. Consequently, analytically demanding fields like metabolomics have particular needs for liquid chromatographic (LC) methods that have the ability to adequately retain and separate polar

Correspondence: Dr. Wolfgang Bicker, Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringer Strasse 38, A-1090 Vienna, Austria
E-mail: wolfgang.bicker@univie.ac.at
Fax: +43-1-4277-9523

Abbreviations: Et(OH)$_2$, 1,2-ethanediol; **HILIC,** hydrophilic interaction chromatography; **ME,** 2-mercaptoethanol; **MEO,** oxidised 2-mercaptoethanol; **MeOH,** methanol; **TG,** 1-thioglycerol; **TGO,** oxidised 1-thioglycerol

* Dedicated to Professor Frantisek Svec on the occasion of his 65th birthday.

** Additional corresponding author: Professor Wolfgang Lindner; E-mail: wolfgang.lindner@univie.ac.at.

solutes under biofluid compatible and mass spectrometric friendly conditions. In this context, chromatography characterised by the retention of polar compounds on polar sorbents with less polar hydro-organic mobile phases as already employed in the 1970s for carbohydrate analysis on amino phases [1] and termed 'hydrophilic interaction chromatography' (HILIC) in 1990 by Alpert [2] was somehow rediscovered within the last few years [3, 4].

The renaissance of HILIC is well reflected in a broad range of bioanalytical applications. Despite this increasing popularity there is still quite limited conclusive information available regarding the actual retention mechanisms. Instead, the early hypothesis that partitioning of the analytes between a water-enriched layer generated on the polar stationary phase and the less polar usually ACN-rich aqueous mobile phase is the driving force in the HILIC retention process is, although at that time formulated with great care by Alpert [2], widely propagated [3, 4]. Earlier results from a critical evaluation of the literature [4] as well as later experimental data not included in this review from 2006 [5 – 16] provide, however, accumulating evidence that partitioning alone can often not satisfactorily explain the chromatographic behaviour of analytes under HILIC conditions.

Indeed, adsorptive polar interactions of solutes with the ligand attached to the sorbent surface or with the sorbent surface itself (*e.g.* silanol groups) can be easily imagined to be superimposed upon the partitioning process. For ligands and analytes that are both charged under the chosen elution conditions long-range electrostatic forces may even become the main retention increment in the HILIC mode. Dissecting individual contributions of 'hydrophilic' and 'ionic' interactions is then often difficult [5, 6, 17, 18]. Furthermore, hydrogen bonding and other dipole – dipole forces may be established between noncharged solute/ligand functionalities and they may potentially contribute to retention and selectivity of (neutral) analytes on chromatographic materials derived from silica and modified with nonchargeable polar ligands [12, 19, 20].

Recently, we reported on the development of noncharged bonded silica materials with different phase polarities and their chromatographic evaluation in RP and HILIC mode [12]. These chromatographic materials are based on vinylised and silanol-endcapped silica on which either 2-mercaptoethanol (ME packing) or 1-thioglycerol (TG packing) is covalently attached (Fig. 1). By on-phase oxidation which converted the embedded sulphide group to the corresponding sulphoxide the more polar oxidised 2-mercaptoethanol (MEO) and oxidised 1-thioglycerol (TGO) packings were yielded (Fig. 1).

The present study was devised to (i) characterise the potential of these novel packings for providing new selectivity increments in HILIC as compared to other silica-based phases (bare silica, diol-type phases) and (ii) extend the tools available for dedicated optimisation of HILIC separations to a series of uncommon protic modifiers in ACN-based mobile phases, complemented by evaluations of salt and temperature effects. 1,2-Ethanediol ($Et(OH)_2$), methanol (MeOH) and ethanol (EtOH) were probed as potential substitutes for the water fraction in the HILIC eluent and nucleobases, nucleosides, as well as deoxynucleosides were used as test solutes (Fig. 2). Besides useful information for HILIC method development the obtained data set was deemed particularly helpful to allow describing and understanding better the scarcely explored mechanisms underlying HILIC separations.

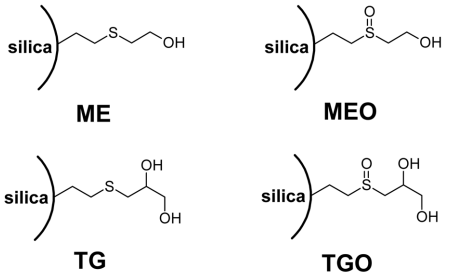


Figure 1. Structural formulas of the home-made stationary phases; adapted from [12].

Conceptually, the term hydrophilic refers to the affinity of molecules to water. Yet, in a broader range it may cover affinity to polar solvents or polar groups in general (http://goldbook.iupac.org (accessed June 30th, 2008)). The acronym HILIC may therefore not be strictly limited to water-containing eluents but includes the use of polar nonaqueous (NA) mobile phases although such elution conditions are frequently denoted as 'polar-organic' mode [21 – 24]. For matter of clarity the terms 'AQ-HILIC' – to describe chromatography performed with hydro-organic mobile phases – and 'NA-HILIC' – which describes chromatography making use of protic organic modifier solvents instead of water – are used in the following.

2 Experimental

2.1 Chemicals and materials

ACN, MeOH and EtOH were of HPLC grade and were supplied by VWR International (Vienna, Austria). $Et(OH)_2$ and ammonium acetate were of analytical grade and purchased from Sigma – Aldrich (Vienna, Austria). Water was bidistilled in-house. 150 × 4 mm id columns packed with the home-made neutral-bonded stationary phases ME, MEO, TG and TGO (Fig. 1) which were all based on 5 μm Daisogel (Daiso Chemical, Osaka, Japan) with 120 Å pore size and a surface area of 300 m^2/g were available from an earlier study [12]. Additional 150 × 4 mm id columns were packed with ProntoSil Diol (5 μm, 120 Å, 300 m^2/g)

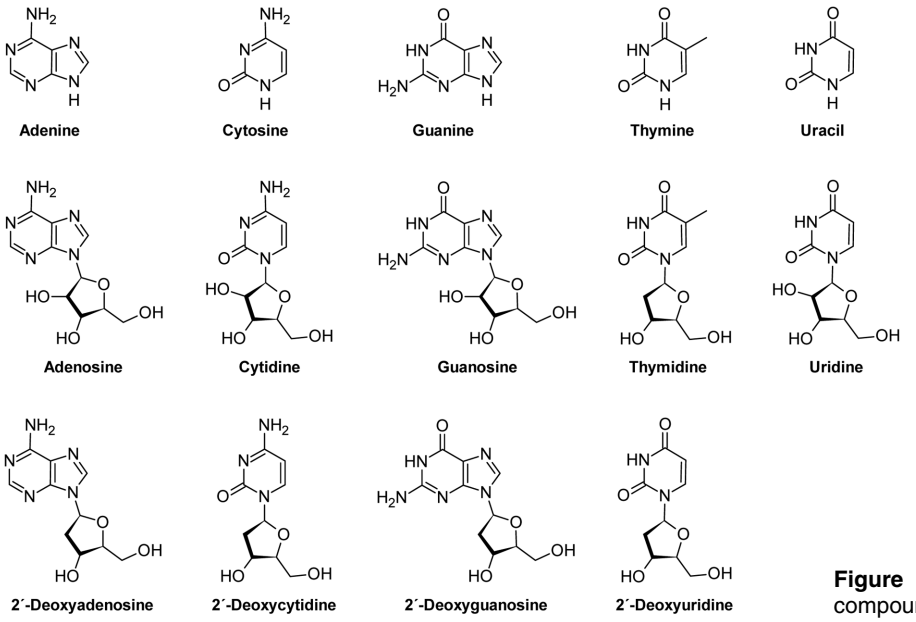


Figure 2. Structural formulas of the test compounds.

(Bischoff Chromatography, Leonberg, Germany) and bare Daisogel, respectively. A Luna HILIC column (5 μm, 200 Å, 200 m^2/g, 150 × 4.6 mm id) originated from Phenomenex (Aschaffenburg, Germany). Chromatographic test compounds (nucleobases, nucleosides, deoxynucleosides) were purchased from Sigma – Aldrich.

2.2 Chromatographic set-up

Chromatographic runs were performed on a 1200 series HPLC system from Agilent (Waldbronn, Germany) equipped with a diode array detector. Data processing was carried out with the HP^{3D} Chemstation software. The flow rate was set at 1.00 mL/min. The column compartment was kept at 25°C (temperature study: 10 – 60°C). The detection wavelength was set at 254 nm. 5 μL fractions of 0.02 – 0.1 mg/mL solutions of the analytes in ACN/water (90:10 v/v) were injected. Void times were measured with toluene. Standard eluents were obtained by dissolving ammonium acetate at a concentration level of 50 mM in either water, or $Et(OH)_2$, or MeOH, or EtOH ('protic modifiers') and mixing these solutions with ACN in a volume ratio of 10 to 90 so that the final salt concentration amounted to 5 mM (no further pH adjustment). Additional mobile phases were prepared to study the chromatographic effects of the amount of MeOH (5 – 30% v/v in ACN, constant amount of 5 mM ammonium acetate) and salt (1 – 15 mM ammonium acetate in ACN/water (90:10 v/v) and ACN/MeOH (90:10 v/v), respectively) in the eluent. Upon a change in the mobile phase composition columns were allowed to equilibrate for a period of 60 column volumes prior to starting analysis runs (acceptance criterion for stable retention times ≤3%RSD).

3 Results

3.1 Effects of type of polar bonding

In a first experimental series using either water (AQ-HILIC mode) or MeOH (NA-HILIC mode) as protic modifier in an eluent composed of 5 mM ammonium acetate in ACN/modifier (90:10 v/v) retention of nucleobases and nucleosides was mapped on the four home-made chromatographic materials. Due to the known structure of the employed noncharged polar ligands (Fig. 1) and comparable surface coverage [12] direct comparisons of the chromatographic effects caused by the type of surface modification were possible.

Figure 3 depicts ln – ln plots of retention factors k of nucleobases and nucleosides as obtained on the ME, MEO, TG and TGO phases when operated in AQ-HILIC and NA-HILIC mode, respectively. Thereby the dotted 45° line represents equivalence in the retention factors k of the plotted stationary phases. A point lying above this line indicates a stronger retention of the respective solute on the phase plotted in y scale while a point below is tantamount to a lower retention.

As expected, MeOH exerted a lower elution strength compared to water which in the NA-HILIC mode caused the analytes to scatter over a wider retention time window. Elution orders within the selected sets of nucleosides and nucleobases, respectively, were maintained on the various stationary phases ($k^{thymine} \sim k^{uracil} < k^{adenine} < k^{cytosine} < k^{guanine}$; $k^{thymidine} < k^{uridine} < k^{adenosine} < k^{cytidine} < k^{guanosine}$) and they did not change upon switching from the AQ-HILIC to the NA-HILIC eluent. Co-elution was observed occasionally such as for thymine and uracil on all phases except on the TGO phase under AQ-HILIC conditions.

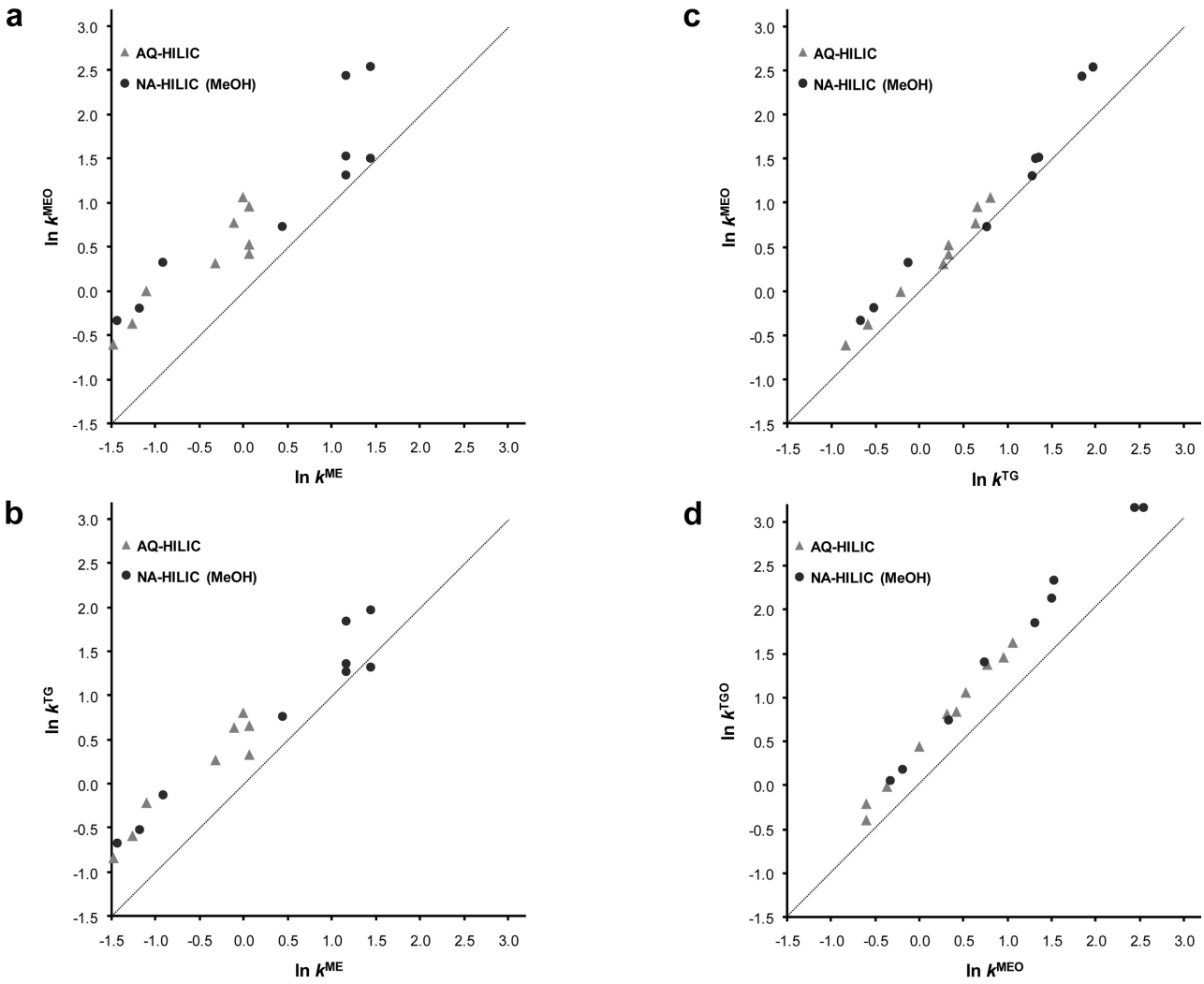


Figure 3. Orthogonality plots of retention factors (ln *k vs.* ln *k*) of home-made stationary phases in AQ-HILIC (water as modifier) and NA-HILIC (MeOH as modifier) elution mode: (a) MEO *versus* ME, (b) TG *versus* ME, (c) MEO *versus* TG, (d) TGO *versus* MEO. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Note: Each point represents either a nucleobase (thymine, uracil, adenine, cytosine and guanine) or a nucleoside (thymidine, uridine, adenosine, cytidine and guanosine), elution orders are given in the text.

The increase in the ligand polarity by exchange of the embedded sulphide through a sulphoxide group (ME *vs.* MEO; Fig. 3a) as well as substitution of the hydroxyethyl by a dihydroxypropyl functionality (ME *vs.* TG; Fig. 3b) in the ligand structure was accompanied by higher *k* values for all analytes. The former group provided a larger retention increment than the latter so that *k* factors were higher on MEO than on TG (Fig. 3c). Consequently TGO, which combined the diol motif of TG with the embedded sulphoxide functionality of MEO, delivered the highest *k* values for all analytes and retention enhancement factors up to 5.6-fold compared to ME (Fig. 3d). Based on this initial column screening retention properties of the developed chromatographic materials followed the order ME < TG < MEO < TGO and this is in reversed order to the RP retention capabilities characterised earlier [12]. This trend held true for both AQ-HILIC and NA-HILIC elution conditions.

Yet, the retention of the individual analytes responded to a different extent to a change in the elution mode and the type of stationary phase, respectively. This resulted in veritable differences in selectivity profiles (Fig. 4). For instance, a much higher purine base selectivity between guanine and adenine was observed on MEO and TGO in the NA (*e.g.* $\alpha^{TGO} = 3.70$) than in the aqueous elution mode ($\alpha^{TGO} = 1.85$) while selectivity values themselves as well as their gain in NA-HILIC mode were considerably smaller on ME and TG (Fig. 4a). This trend was also obeyed by the corre-

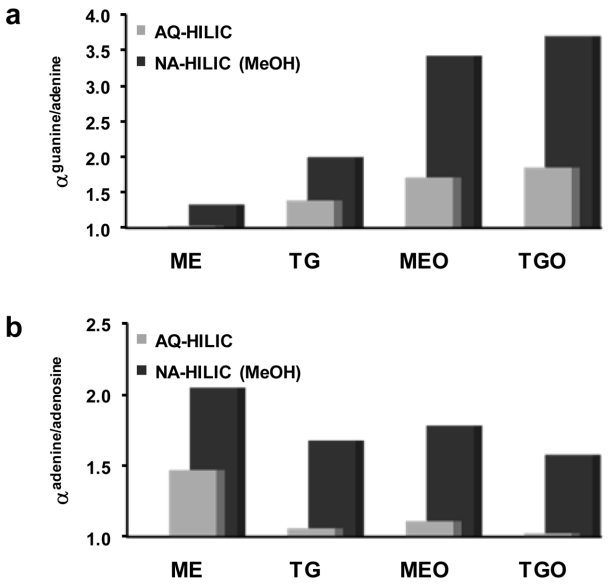


Figure 4. (a) Guanine/adenine and (b) adenine/adenosine selectivities of the developed packings in AQ-HILIC (water as modifier) and NA-HILIC (MeOH as modifier) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2.

sponding nucleoside pair. On the other hand, the highest adenine/adenosine selectivity (nucleobase/nucleoside) was observed on the nonoxidised ME packing (Fig. 4b).

To closer examine chromatographic effects caused by the presence of sulphoxides in polar bondings as opposed to packings lacking such a functionality subsequent studies were carried out with the TGO, the TG and a few commercial HILIC columns.

3.2 Effects of type of protic modifier

Figure 5 depicts chromatograms of nucleobases and nucleosides as obtained on the TG, the TGO and the bare silica column, respectively with four different types of protic modifiers in the ACN-based eluent, namely water, $Et(OH)_2$, MeOH and EtOH.

Elution orders within the sets of nucleosides and nucleobases, respectively were analogous on TG and TGO and in most cases nucleosides were stronger retained than their corresponding nucleobases. Some solvent specific effects were also present, as for example indicated by a reversal of the nucleobase pair adenine and cytosine in case of using $Et(OH)_2$ as protic modifier. The elution order observed on the bare Daisogel silica column in AQ-HILIC mode and in NA-HILIC mode with MeOH largely resembled that of the modified phases (Fig. 5c). Only cytidine and cytosine, respectively, were each stronger retained than guanosine and guanine. In case of $Et(OH)_2$ the nucleoside elution order was identical to that obtained with TG and TGO. Noteworthy, on the silica packing the use of the weak eluting protic modifier EtOH deteriorated peak shapes of all nucleosides except the first eluting thymidine (Fig. 5c) and cytidine as well as guanosine could not be detected.

Retention factors on TG and TGO increased as expected in the order H_2O < $Et(OH)_2$ < MeOH < EtOH. The enhancement of retention in the NA-HILIC mode *versus* AQ-HILIC conditions was usually more pronounced on the more polar TGO packing. Some peculiarities in this respect were, however, observed for $Et(OH)_2$. Retention of nucleobases tended to respond stronger to a change from aqueous to NA elution conditions than their corresponding nucleosides particularly when MeOH or EtOH were used as modifiers. Interestingly, on the bare silica column $Et(OH)_2$ was found to be a slightly stronger eluting protic modifier than water for all nucleosides and the nucleobases uracil and cytosine (for thymine, thymidine, uracil and uridine also MeOH was found to be a stronger eluting additive than water).

Significant solvent effects and inter-column differences were accordingly observed for chromatographic selectivities (Figs. 6 and 7). For instance, adenosine/uridine (purine/pyrimidine) nucleoside selectivity increased in the order TGO < TG < silica (Fig. 6a). In contrast, the order of selectivity for guanosine/adenosine (purine/purine) changed to silica < TG < TGO (Fig. 6b) and for cytidine/adenosine to TG < TGO < silica (Fig. 6c). In all cases, a lower polarity of the protic modifier translated into higher selectivities. For the pyrimidine nucleoside/nucleobase pair uridine/uracil neither a change of the stationary phase nor the type of modifier exerted major effects on selectivity (Fig. 7a). On the other hand, adenine/adenosine selectivity was more susceptible to a change in the type of protic modifier (Fig. 7c) (note the inversed elution order for this compound pair, *i.e.* nucleoside before nucleobase).

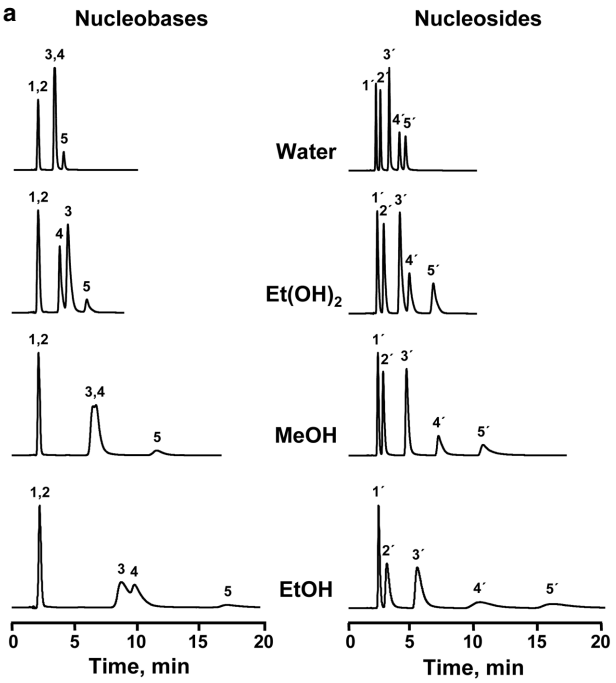
To figure out similarities and dissimilarities between our novel packings and established neutral but polar modified phases of diol-type, comparative investigations were carried out with Luna HILIC and ProntoSil Diol. As shown in Fig. 8 elution orders of both of these columns largely resembled those observed on TG and TGO including the reversal of the elution order of adenine and cytosine in the NA-HILIC mode using $Et(OH)_2$ as modifier. On the Luna HILIC column also MeOH led to such a reversed elution order while with EtOH co-elution of these two solutes was observed. In the AQ-HILIC mode all nucleosides not including adenosine were stronger retained than their respective nucleobases and such a pattern was mostly also observed for NA elution conditions.

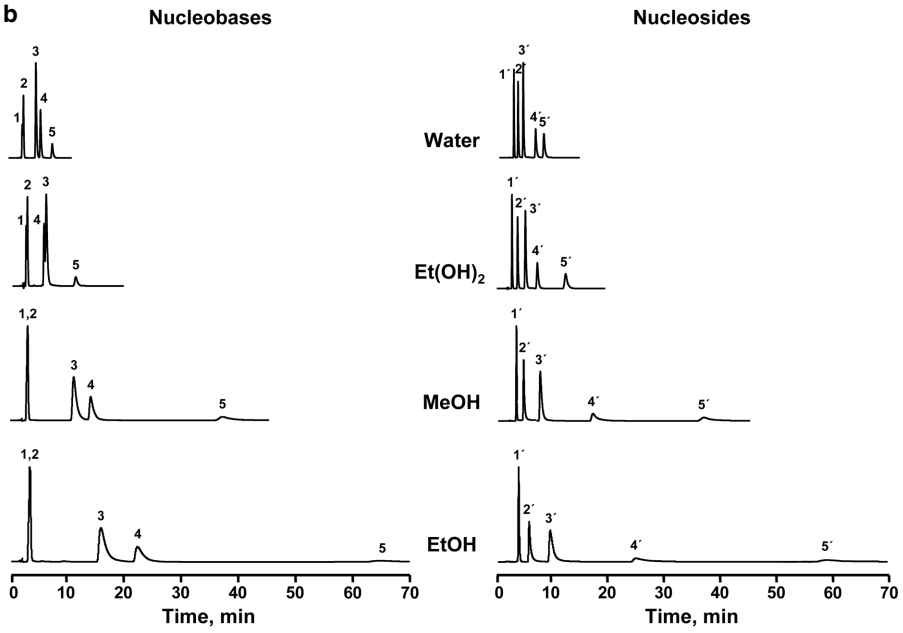
In analogy to the TG and TGO packing a protic modifier eluotropic strength order of EtOH < MeOH < $Et(OH)_2$ < H_2O could be established for Luna HILIC and ProntoSil Diol. The gain in retention upon reducing the polarity of

the protic modifier was usually stronger for nucleobases although the general trend of increasing retention upon reducing the polarity of the modifier was not that evident for all solutes. On both packings the early eluting solutes thymidine and uridine were slightly stronger retained in the AQ-HILIC mode compared to NA conditions and cytosine as well as cytidine experienced a weaker retention in the NA-HILIC mode when $Et(OH)_2$ was used as modifier.

Trends in changes of selectivity for adenosine/uridine, guanosine/adenosine, as well as cytidine/adenosine as a function of the type of modifier were for the two commercial diol-type columns (Fig. 9) in good agreement with those observed with the TG and TGO packings (Fig. 6). Thereby, absolute α values seemed to match better with the diol but not sulphoxide containing TG phase. Nucleoside/nucleobase selectivity patterns of the diol-type columns as a function of the various modifiers (Fig. 10) were also related to that of the home-made bonded packings TG and TGO (Fig. 7).

To further exemplify the practical usefulness of switching from aqueous to NA elution conditions for solving a





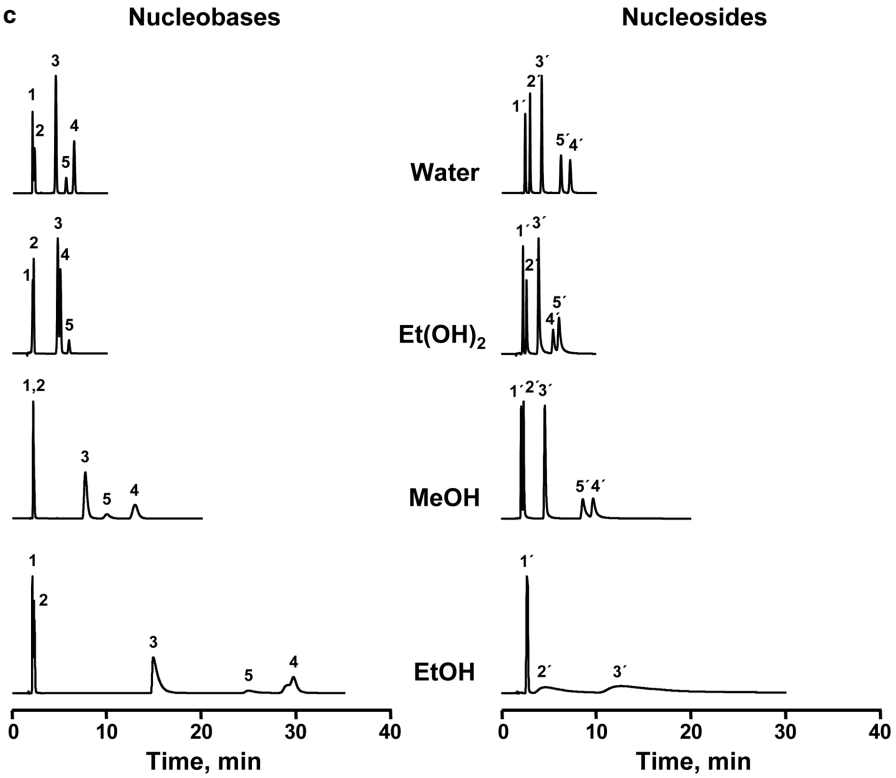


Figure 5. Separation of the sets of nucleobases and nucleosides on (a) the TG, (b) the TGO and (c) the bare silica (Daisogel) packing in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Analytes: (1) thymine, (2) uracil, (3) adenine, (4) cytosine, (5) guanine; (1′) thymidine, (2′) uridine, (3′) adenosine, (4′) cytidine and (5′) guanosine.

separation problem with polar solutes a mixture composed of four nucleosides and corresponding 2′-deoxynucleosides was analysed. For this purpose Luna HILIC and the home-made TGO packing were selected and they were operated with the standard AQ-HILIC eluent as well as with MeOH and $Et(OH)_2$ containing NA mobile phases, respectively.

The set of nucleosides and the set of deoxynucleosides were each well separated on these columns both in the AQ-HILIC as well as in the NA-HILIC mode (for nucleoside chromatograms see Figs. 5b and 8a). However, when all of these solutes were analysed simultaneously critical selectivity situations emerged (Fig. 11): (i) aqueous elution conditions failed to provide baseline separation between 2′-deoxyguanosine (compound 5*) and cytidine (compound 4′) on both columns and (ii) adenosine (compound 3′) and 2′-deoxycytidine (compound 4*) co-eluted on Luna HILIC when $Et(OH)_2$ was used as modifier. Thus, on the home-made TGO packing all eight analytes could already be resolved by substituting water in the eluent by $Et(OH)_2$. Yet, a prolonged total run time of around 12 min had to be accepted for this improved separation compared to around 8 min in the AQ-HILIC mode. On the commercial Luna HILIC column only the use of MeOH as protic modifier delivered baseline separation of all solutes (increase of the run time from 8 min to 15 min). For the given separation problem it was further proven that in AQ-HILIC under isoelutropic conditions, as adjusted for 2′-deoxyguanosine by a reduction of the water content to yield a k value that was similar to that obtained in the NA-HILIC mode, still not all compounds could be baseline separated. This is one illustrative example for true solvent-mediated chromatographic effects of the employed NA-HILIC elution conditions.

In practical use the selectivity benefit that might arise due to the choice of NA-HILIC conditions has to be well balanced with the potential need for longer analysis times and the risk for loss of peak efficiency. Effective plate heights (H_{eff}) tended to increase in the order $Et(OH)_2 \sim$ water < MeOH < EtOH being significantly worse only for EtOH. H/u-plots as exemplarily studied with Luna HILIC provided optimum flow rates of 0.5 – 0.75 mL/min. Peak efficiency studies on intercolumn basis were, however, limited by the fact that for the different in-house prepared columns a nonspecifically optimised packing protocol was used for column preparation. Thus peak shape differences as may be appraisable from Figs. 5, 8 and 11 have to be interpreted with care. Although not yet studied in detail also the use of a water-containing injection solvent (due to limited solubility of most solutes under NA conditions) may act as a peak shape influencing parameter under the various elution conditions [25].

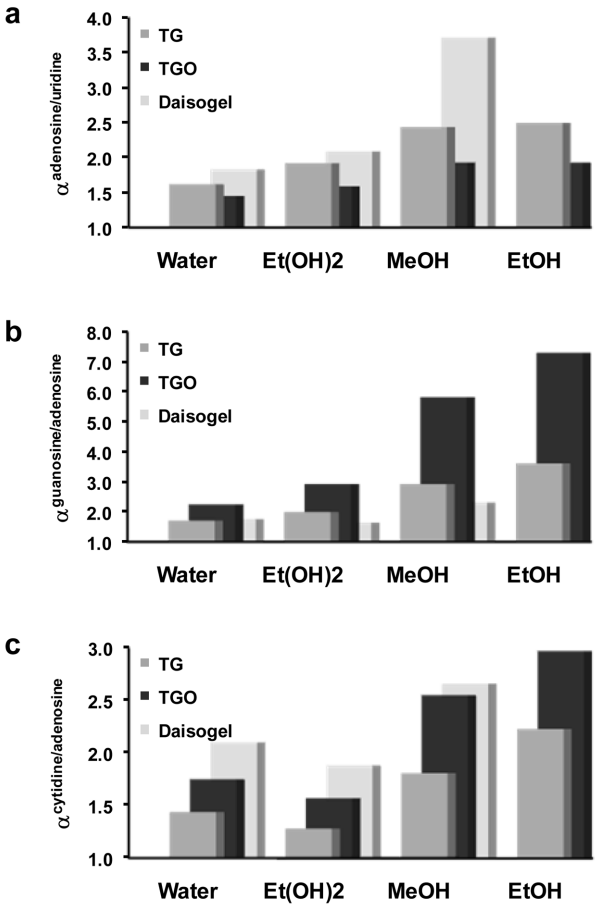


Figure 6. (a) Adenosine/uridine, (b) guanosine/adenosine and (c) cytidine/adenosine selectivities of the TG, the TGO and the bare silica (Daisogel) packing in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Note: Due to defective nucleoside peak shapes on the bare silica column with EtOH as modifier (*cf.* Fig. 5) accurate selectivities for this packing could not be determined and are therefore not shown.

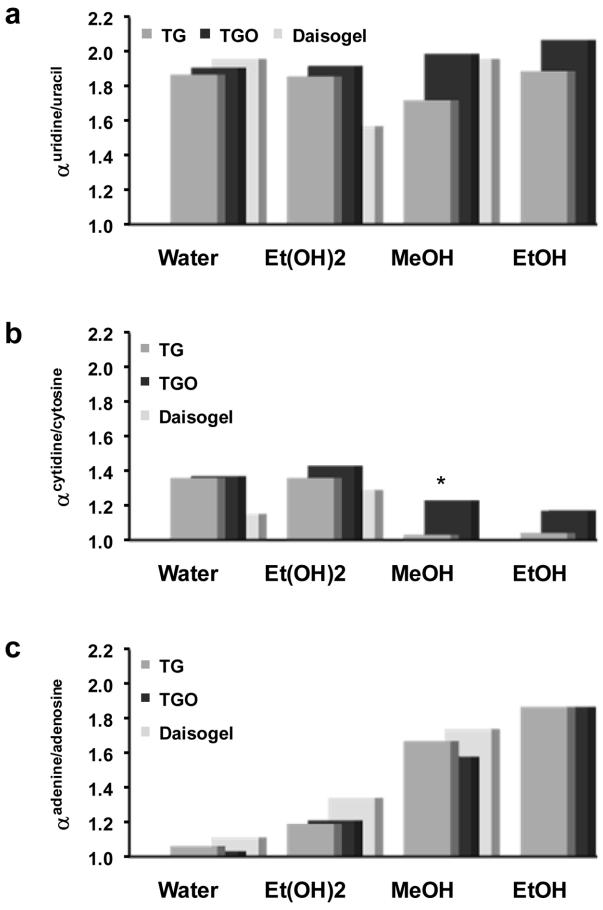


Figure 7. (a) Uridine/uracil, (b) cytidine/cytosine and (c) adenine/adenosine selectivities of the TG, the TGO and the bare silica (Daisogel) packing in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Note: Due to defective nucleoside peak shapes on the bare silica column with EtOH as modifier (*cf.* Fig. 5) accurate selectivities for this packing could not be determined and are therefore not shown. * Reversal of elution order on bare silica.

3.3 Effects of protic modifier amount in NA-HILIC mode

The amount of water in the eluent plays a primary role for adjustment of retention in the AQ-HILIC mode [3, 4]. In analogy, the polar solvent percentage in the NA-HILIC mode was investigated for MeOH and the TGO stationary phase.

As expected, k values dropped with the amount of MeOH in ACN (tested range of 5 – 30% v/v MeOH, constant amount of 5 mM ammonium acetate). A linear dependence of the relationship between k and the volume fraction of MeOH in the eluent was found in the log – log scale (r^2 >0.99) whereas in the log – lin scale a significant curvature of such plots occurred (Fig. 12).

Stronger retained compounds responded to a change in the MeOH content to a higher degree. For example, slope values of the linear regression lines obtained in the log – log plot for nucleosides increased in the order adenosine (slope −1.23) < cytidine (slope −1.68) < guanosine (slope −1.83). With regards to guanosine and cytidine slopes were higher as opposed to the corresponding nucleobases ($slope^{cytosine}$ −1.48, $slope^{guanine}$ −1.68). This is in agreement with earlier findings in the AQ-HILIC mode [12]. For adenosine the dependence of retention on the MeOH content was slightly lower than for adenine.

Chromatographic selectivity was also affected by a change in the MeOH concentration. For example, the α value of guanosine/adenosine decreased from 8.2 at 5%

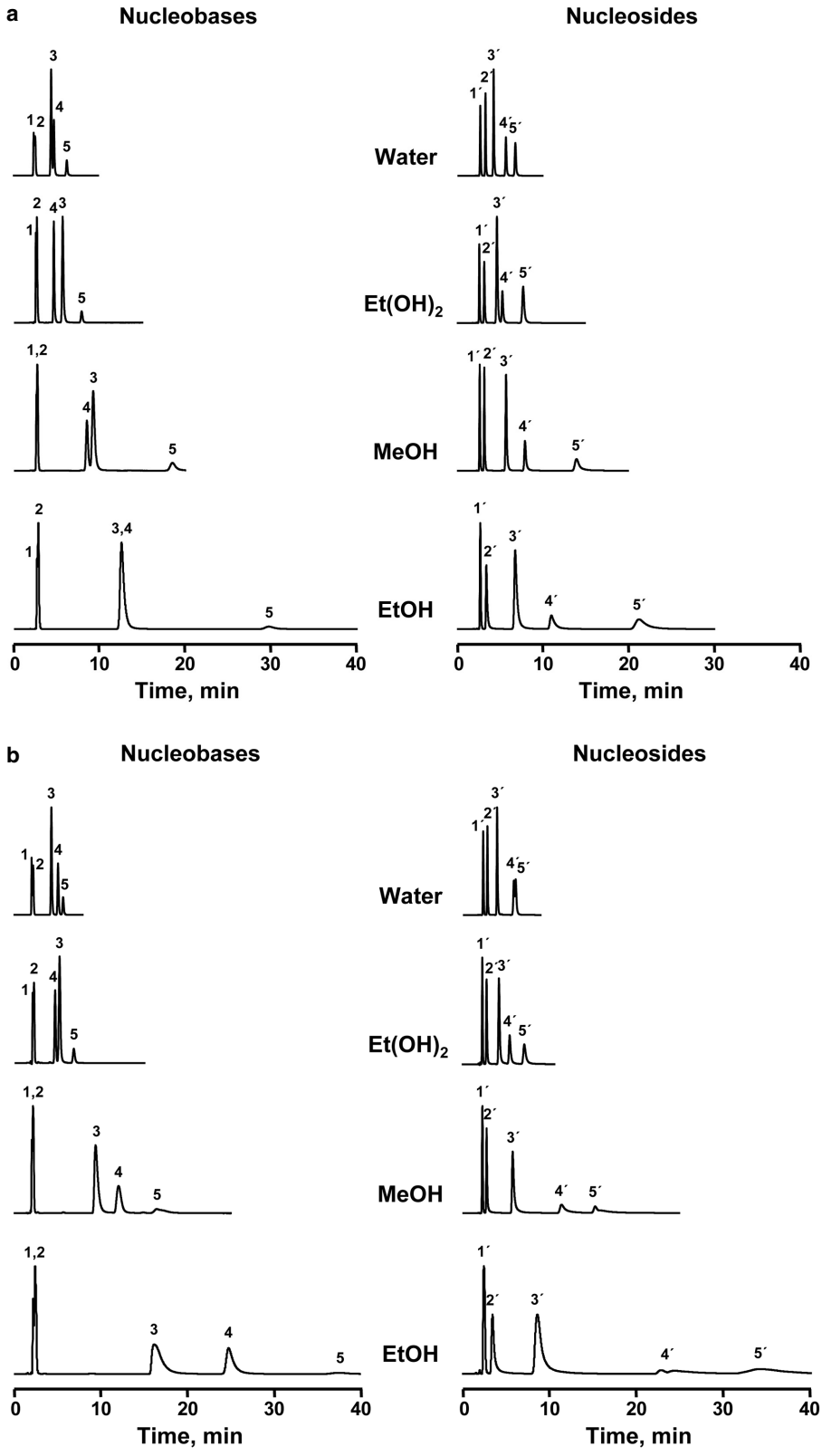


Figure 8. Separation of the sets of nucleobases and nucleosides on (a) Luna HILIC and (b) Prontosil Diol in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Analytes: (1) thymine, (2) uracil, (3) adenine, (4) cytosine, (5) guanine; (1′) thymidine, (2′) uridine, (3′) adenosine, (4′) cytidine and (5′) guanosine.

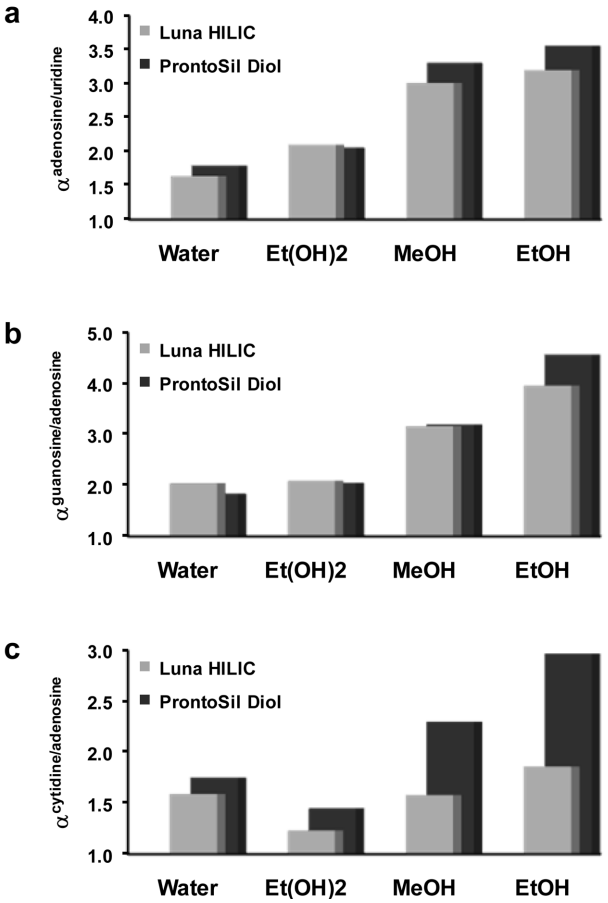


Figure 9. (a) Adenosine/uridine, (b) guanosine/adenosine and (c) cytidine/adenosine selectivities of Luna HILIC and ProntoSil Diol in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2.

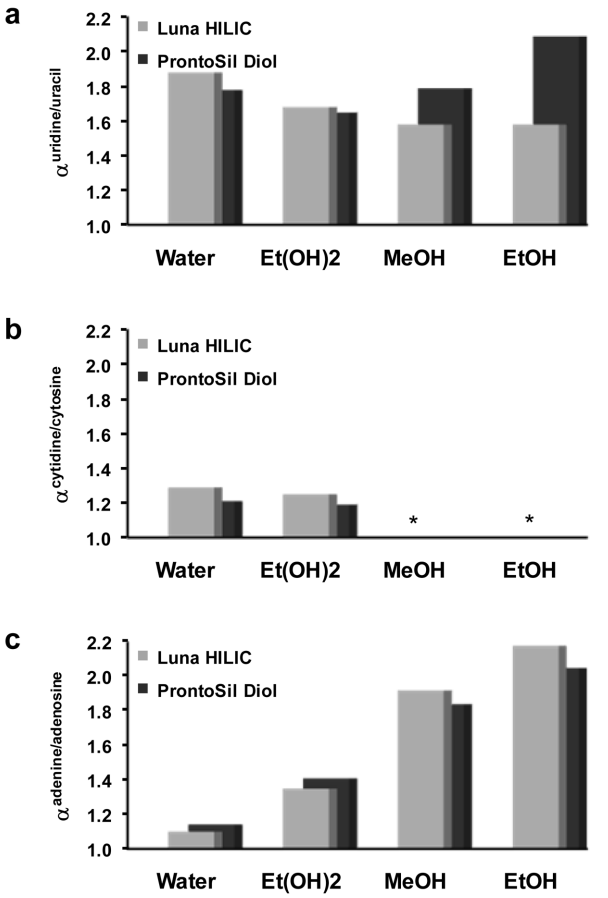


Figure 10. (a) Uridine/uracil, (b) cytidine/cytosine and (c) adenine/adenosine selectivities of Luna HILIC and ProntoSil Diol in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. * Reversal of elution order on both packings.

MeOH in the eluent to 2.8 when the MeOH content was raised to 30%. Isoelutropic conditions in AQ- and NA-HILIC modes (with regards to k of adenosine) made solvent-specific selectivity effects particularly obvious ($\alpha^{\text{NA-HILIC (15\% MeOH)}} = 4.40$, $\alpha^{\text{AQ-HILIC (10\% water)}} = 2.25$). The selectivity of guanine/adenine was affected in a similar manner by the MeOH content while the overall decrease within the studied MeOH concentration range was just around 50% (from 4.6 to 2.2; AQ-HILIC (10% water): $\alpha = 1.85$). On the other hand, absolute nucleoside/nucleobase selectivities were affected to a lesser extent (*e.g.* $\Delta\alpha^{5 \rightarrow 30\% \text{ MeOH}}$ (adenine/adenosine) = −0.08) although for certain pairs (*e.g.* guanosine/guanine, cytidine/cytosine) reversals of elution orders were observed (*cf.* Fig. 12).

3.4 Effects of salt concentration

The role of salt in the HILIC retention process was studied with three different concentration levels of ammonium acetate (1, 5 and 15 mM) in both the AQ-HILIC and the NA-HILIC mode. For NA elution conditions MeOH was selected as modifier. Let us first discuss the results of the AQ-HILIC mode.

An increase of the amount of salt in the AQ-HILIC eluent caused the solutes to elute later except for the retention of adenine on TGO and bare silica. The extent of this salt effect on retention depended, however, strongly on the types of analyte and column. Increasing the salt load from 1 to 15 mM enhanced for example the retention of cytidine by 33% on TGO while this value was 80% on both TG and Luna HILIC, 105% on ProntoSil Diol, and even 140% on the bare silica packing (Fig. 13). In contrast to that, the retention of cytosine responded to a minor degree (7% on TGO, 42 – 45% on the four other columns). With respect to the column type the gain in retention of both nucleosides and nucleobases largely followed the order TGO < Luna HILIC ~ TG < ProntoSil Diol < bare

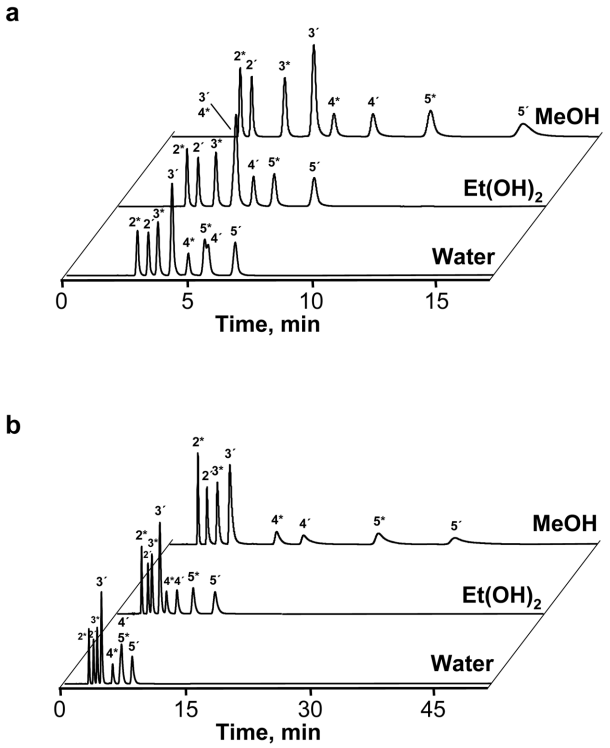


Figure 11. Separation of nucleosides and deoxynucleosides on the (a) Luna HILIC and (b) TGO column in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$ and MeOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2. Analytes: (2′) uridine, (3′) adenosine, (4′) cytidine, (5′) guanosine; (2*) 2′-deoxyuridine, (3*) 2′-deoxyadenosine, (4*) 2′-deoxycytidine and (5*) 2′-deoxyguanosine.

silica, *i.e.* the type of bonding and/or the ligand surface coverage seemed to have a prime influence on the extent of the salt effect.

In the NA-HILIC mode a more diverse dependency of retention on the salt concentration was observed. Some of the analytes showed a pronounced enhancement of k at higher salt concentrations (similar to AQ-HILIC) while other solutes experienced weaker retention. For example, the change in retention of cytidine varied between −27 and +46% upon increase from 1 to 15 mM ammonium acetate (Fig. 13). In contrast, a negative effect on retention existed generally for cytosine (range from −52 to −31%), while guanosine was stronger retained (12–89%) when 15 instead of 1 mM ammonium acetate were present in the NA eluent.

Owing to the different susceptibilities of the analytes towards a change in the salt concentration chromatographic selectivity was also affected by this mobile phase parameter. For instance, nucleoside/nucleobase selectivity increased with higher salt concentrations (*e.g.* TGO in AQ-HILIC mode: $\alpha^{\text{cytidine/cytosine}}$ (1 mM) = 1.26, $\alpha^{\text{cytidine/cytosine}}$ (15 mM) = 1.56) which in the NA-HILIC even led to reversals of elution order for some pairs at low amounts of salt (*i.e.* nucleobase was stronger retained than nucleoside). With regards to nucleoside selectivity both enhancement (*e.g.* guanosine/adenosine) and decrease (*e.g.* adenosine/uridine) of α values was observed upon increasing the amount of salt in the eluent (Fig. 14). From a practical point of view a dedicated optimisation of the salt concentration helped for example to improve chromatography for the adjacent peaks cytidine/guanosine on ProntoSil Diol in the AQ-HILIC mode from α = 1.00 (co-elution) at 1 mM ammonium acetate to baseline separation with α = 1.09 at 15 mM.

3.5 Effects of temperature

Column compartment temperature is known to be an important influential factor for retention and selectivity in the AQ-HILIC mode [26]. Presently, comparative investigations in the AQ-HILIC and in the NA-HILIC mode were performed with the TG and TGO columns in a temperature range of 10–60°C to elucidate differences between packings, modifiers, and individual analytes.

Regardless of the type of protic modifier all analytes showed a loss of retention upon increasing the column

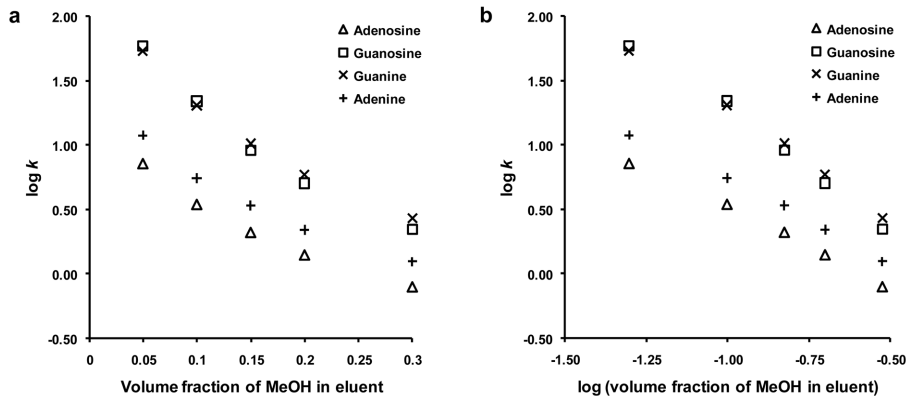


Figure 12. Plots of (a) log k *versus* volume fraction of MeOH in the NA-HILIC eluent and (b) log k *versus* log (volume fraction of MeOH in the NA-HILIC eluent) of adenine, guanine, adenosine and guanosine as obtained on the TGO packing. Mobile phases: ACN/MeOH mixtures of varying MeOH fractions (5–30% v/v) and a constant amount of 5 mM ammonium acetate. Further chromatographic details are given in Section 2.

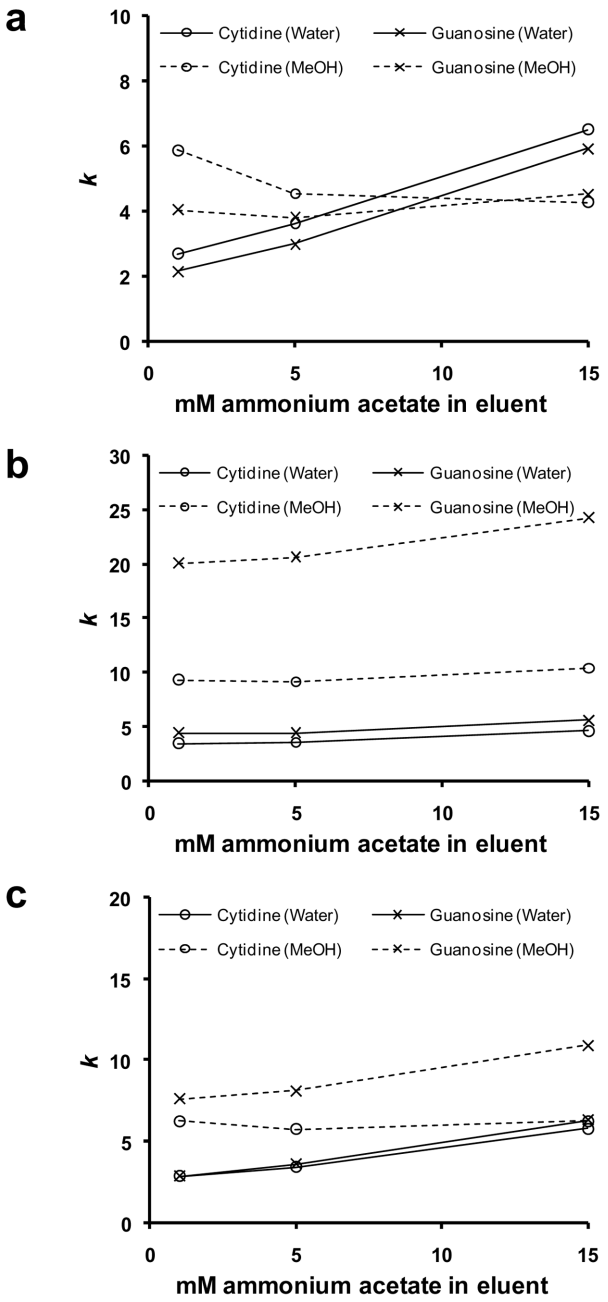


Figure 13. Retention factors *k* of cytidine and guanosine on (a) bare silica (Daisogel), (b) TGO and (c) ProntoSil Diol as a function of the amount of ammonium acetate in the AQ-HILIC (water as modifier; solid lines) and NA-HILIC (MeOH as modifier; dashed lines) eluent, respectively. Mobile phase: 1 – 15 mM ammonium acetate in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2.

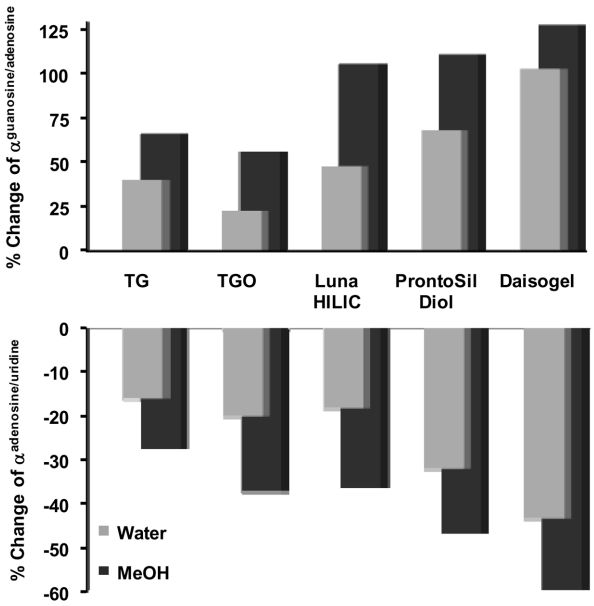


Figure 14. Change (%) in selectivities of guanosine/adenosine and adenosine/uridine on various packings as a function of increasing amounts of ammonium acetate (15 *vs.* 1 mM) in the AQ-HILIC (water as modifier) and NA-HILIC (MeOH as modifier), respectively eluent. Mobile phases: 1 and 15 mM ammonium acetate, respectively in ACN/modifier (90:10 v/v). Further chromatographic details are given in Section 2.

compartment temperature. Yet, the effects on selectivity were more complex (Fig. 15). As shown in Fig. 15a separations of the compound pairs guanine/cytosine on TGO with mobile phases containing either water or $Et(OH)_2$ as protic modifiers were found to retain almost identical selectivity values over the entire investigated temperature range while with MeOH or EtOH selectivities diminished considerably at higher temperature. Such a pronounced temperature effect for the latter modifiers in comparison to water and $Et(OH)_2$ was not present for other compound pairs, *e.g.* guanosine/cytidine (Fig. 15b).

Nucleoside/nucleobase selectivity was subject to significant temperature effects as well. Here separations obtained with the more polar modifiers water and $Et(OH)_2$ were somehow stronger influenced by a change of the temperature compared to the aforementioned nucleoside/nucleoside and nucleobase/nucleobase selectivities, respectively. In case of using the less polar modifiers MeOH or EtOH an increase in the temperature resulted also in a decrease of selectivity whereby reversals of elution orders in the range of 30 – 50°C were observed for example for cytidine/cytosine on both packings, *i.e.* at higher temperatures the nucleobase was stronger retained than the nucleoside.

$1/T$ *versus* $\ln k$ plots showed linearity (r^2 >0.99) for all analytes and modifiers, respectively. Retention enthalphies ΔH calculated according to the van't Hoff equation were negative for each solute (exothermic process) while a distinct trend between the two columns was not recognised. For instance, ΔH values of cytosine on the TG phase varied between −7.6 and −10.4 kJ/mol for the four different modifiers, whereas for its corresponding nucleoside cytidine these values ranged from −10.5 to −12.5 kJ/mol.

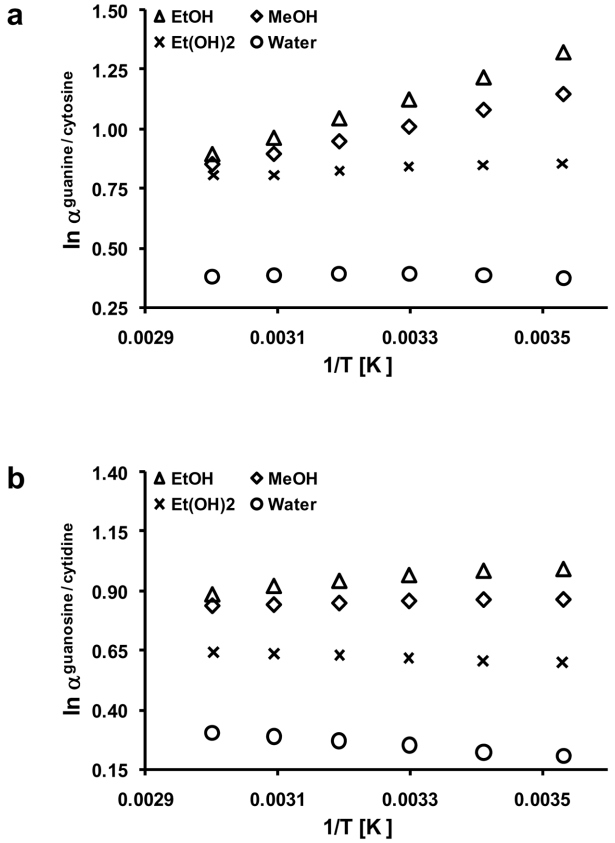


Figure 15. Plots of ln α *versus* $1/T$ [*K*] of (a) guanine/cytosine and (b) guanosine/cytidine as measured with the TGO column in AQ-HILIC (water as modifier) and NA-HILIC ($Et(OH)_2$, MeOH, EtOH as modifiers) elution mode. Mobile phase: 5 mM ammonium acetate in ACN/modifier (90:10 v/v). Column compartment temperature was varied from 10 to 60°C. Further chromatographic details are given in Section 2.

On the more polar TGO packing these figures were −7.5 to −10.8 and −10.2 to −15.2 kJ/mol, respectively. Thereby retention enthalpies adopted in direction more negative values when the polarity of the protic modifier was decreased. In other words retention times in the AQ-HILIC mode were typically less affected by a change in temperature than those obtained under NA-HILIC elution conditions.

Nucleosides delivered more negative ΔH values than their corresponding nucleobases with water and $Et(OH)_2$ as modifiers (*e.g.* TGO: $\Delta\Delta H^{\text{cytidine/cytosine}}$ (water) = −3.5 kJ/mol, $\Delta\Delta H^{\text{guanosine/guanine}}$ (water) = −2.1 kJ/mol). This finding validates our earlier data obtained with these phases with an eluent composed also of 10% water in ACN but containing ten instead of 5 mM ammonium acetate [12]. In case of MeOH or EtOH the ΔH value of the nucleoside was for some compound pairs still considerably more negative (*e.g.* ΔH of cytidine up to 4.7 kJ/mol more negative than ΔH of cytosine) while for others almost identical values were yielded (*e.g.* guanosine/guanine $\Delta\Delta H$ between 0.2 and 0.5 kJ/mol).

4 Discussion

The chromatographic data provided in this article add to the ongoing debate about the HILIC retention mechanisms and are therefore basically examined in the following in attempts to (i) find evidence for adsorptive interactions in the commonly believed partitioning-driven HILIC retention process and (ii) characterise the potential of the various investigated stationary and mobile phase parameters for optimising separation conditions.

The ligands of the employed modified silicas selected for the present study (see Fig. 1 for home-made packings) had in common that all of them contained hydroxy groups (some of them diol motifs) but no functionalities being chargeable under the chosen elution conditions. Additionally, embedded ether (ProntoSil Diol, Luna HILIC) or sulphide (ME, TG) or sulphoxide (MEO, TGO) groups were present. Apart from silanophilic interactions the lack of chargeable functionalities prevented the development of strong electrostatic solute–ligand interactions that may potentially influence the actual HILIC retention process. The selected polar bondings, in conjunction with data obtained with the bare silica material, should thus be particularly useful for interpreting the results in view of 'actual' HILIC interactions.

4.1 Effects of type of polar bonding

If partitioning is considered to be an important retention mechanism in HILIC the water layer adsorbed on the sorbent surface (potentially in combination with a salt fraction) represents the stationary phase. Indirect evidence that such a layer is indeed present under the chosen elution conditions is provided by an increase of the 'void' time (measured with toluene) when the polarity of the protic modifier was decreased. For example, on the most polar home-made phase TGO this increase was about 10% if comparing the most polar (*i.e.* water) with the least polar (*i.e.* EtOH) protic modifier while for the less polar TG packing this value was just around 6%.

This finding might be taken as an evidence that in the AQ-HILIC mode part of the pore volume is captured by water from which the hydrophobic toluene is excluded. A similar observation was made by McCalley and Neue [27], who noticed pronounced effects on the elution times of benzene and toluene on a bare silica packing upon changing the water fraction in the ACN-based eluent. It was concluded that 4–13% of the entire pore volume might be occupied by water when the amount of ACN in the mobile phase was in the range of 75–90%.

Upon exchanging water by alcohols in our eluent system the supposed aqueous layer should be mainly replaced by an alcohol layer which will likely differ in thickness but is definitely more hydrophobic. This in turn facilitates increased penetration of the pores by toluene thus leading to a greater void time. Along this line it is not surprising that the change of the elution time of toluene is more pronounced with a more polar packing (TGO *vs*. TG) as this helps building up a thicker layer especially in the AQ-HILIC mode. This in turn causes a stronger exclusion effect for hydrophobic solutes. Such subtle effects make it a subject of debate how the void time in AQ- and NA-HILIC can be determined in an accurate manner.

The observed increase in retention of the test compounds on more polar phases in the AQ-HILIC mode (Fig. 3) follows the general trend to be expected by a change of the phase ratio in a partitioning retention model. In such case the thickness of the adsorbed solvent layer for a given elution condition is basically controlled by the polarity of the ligand and the ligand surface coverage. Thus, the relative gain in retention should be somehow uniform among the set of analytes. The considerable scatter of the points depicted in Figs. 3a and b demonstrates, however, that some analytes are more affected by a change of the structure of the ligand immobilised on silica surface than others. This suggests the involvement of adsorptive interactions that add to partitioning processes.

Most prominently it was found that both in the AQ- and in the NA-HILIC mode the introduction of sulphoxides into the ligand structure of the ME packing (MEO phase) exerted distinctly different effects on retention and selectivity than introducing an additional hydroxyl functionality (TG) (Figs. 3 and 4). This held also true in case a sulphoxide group was introduced into a diol-motif containing ligand (TG *vs*. TGO) (Figs. 3 – 7). It was further proven that the employed base Daisogel silica possessed a similar gross elution pattern of the selected solutes but significantly different retention and selectivity properties compared to the various neutral-bonded Daisogel packings. Chromatographic differences, particularly of the sulphoxide-containing TGO packing, were also present as opposed to the tested commercial columns of diol-type (see for example Fig. 11). Yet, direct comparison between our materials and the two established diol-type packings is hampered by the fact that ligand density and the type of base silica likely will have an effect on retention and selectivity as well (recent data suggest, however, a minor influence of the latter [28]).

Interpreting the observed effects on a molecular basis we may ascribe the differential results obtained for the TG and TGO phase to the fact that hydroxy groups may act as both hydrogen bond donors and acceptors whereas the sulphoxide group is solely a hydrogen bond acceptor group (the embedded sulphide group in TG is not considered to deliver substantial hydrophilic retention increments). Similarly, differences in retention profiles of TGO and the selected commercial diol-type phases (which according to the manufacturers both feature incorporated ether functionalities as hydrogen bond acceptor groups) may also be related to the specific kind of polar functionality. We, therefore, assume that the chromatographic ligand participates actively in the retention process and thus the specific types of polar functionalities determine to which extent discriminative interactions with analytes are being formed. Such kind of interpretation is close to the concept of adsorption-driven normal-phase separations.

Yet, it has to be particularly considered that under these polar elution conditions direct solute – ligand interactions may be hampered by the solvation shell which surrounds interactive sites on the sorbent surface and the analyte. Desolvation may be energetically unfavoured under the typical elution conditions but multiple hydrogen bonding of the analyte with adsorbed solvent molecules may nevertheless occur. The extent of such 'indirect' solute – ligand interactions may of course be dictated to a certain extent by the ligand structure as the availability of hydrogen bond donor/acceptor sites will likely affect the microstructure of the solvation network surrounding the ligand. In this context, interactions of the analytes with solvent that is either contained in the solvation shell of polar ligand functionalities and/or tightly bound to the silica surface may conceptually be regarded as being on the smooth transition from 'adsorption' to 'partitioning'. Furthermore, a full deconvolution of adsorptive and partition-related contributions to HILIC retention is also not possible due to the assumed lack of a distinct boundary between bulk mobile phase and the partially dynamically generated stationary phase (adsorbed solvent layer and solvation shells). More likely the protic modifier concentration will gradually decrease by going from the sorbent surface to the mobile phase.

Based on these considerations it is already justified to drop the simple partitioning retention model at least for our phases in favour of a multiple interaction or mixed-mode retention model. Further experimental data supporting this concept are provided in the following.

4.2 Effects of type and amount of protic modifier

It was demonstrated that retention of polar nucleobases and nucleosides can be accomplished on all of the selected chromatographic materials not only under common AQ-HILIC conditions but also in the NA-HILIC mode. With very few exceptions NA-HILIC conditions provided stronger retention and this gain depended besides the type of polar packing mainly on the polarity of the modifier (eluotropic strength increased with modifier polar-

ity) and the type of analyte (retention of nucleobases was usually more enhanced than that of corresponding nucleosides).

Elution orders were largely preserved comparing the AQ-HILIC mode with NA elution conditions which indicates that retention mechanisms of AQ- and NA-HILIC modi are – as would be expected for partitioning processes – to some extent similar. However, distinct solvent effects together with intercolumn differences (Figs. 5 – 11) strongly point towards the involvement of adsorptive interaction increments.

In accordance with our earlier data obtained in the AQ-HILIC mode [12] also under NA-HILIC conditions the retention of the chosen set of nucleosides and nucleobases was maximal when the amount of the protic modifier was minimal. This is to be expected for a typical HILIC retention mechanism [4]. Although the higher eluotropic strength of mobile phases containing higher amounts of protic modifiers translate to a general trend of decreasing retention (Fig. 12) chromatographic selectivities were found to be affected in a more subtle way (*vide supra*). It could for example be demonstrated that the use of higher amounts of MeOH in NA-HILIC eluents to yield isoelutropic conditions compared to the AQ-HILIC mode resulted in veritable selectivity differences. Such findings are strongly supportive for the occurrence of solvent-specific selectivity effects.

From a mechanistic point of view it was interesting to observe that on the home-made TGO packing $\log k$ dropped linearly with the logarithm of the MeOH fraction in the eluent while a significant curvature was observed for a corresponding log – lin plot. An analogous pattern was obtained earlier in the AQ-HILIC mode [12]. Such a dependence of the retention factor on the amount of the stronger eluting solvent fraction in the mobile phase can be associated with a Snyder – Soczewinski-like retention model similar to surface adsorption-driven normal-phase chromatography [29]. If we assume the occurrence of hydrogen bonding or other dipole – dipole interactions in our separation system retention of analytes having a larger number of adsorptive-interactive sites in the molecule (*e.g.* sugar element in nucleosides but not nucleobases) are stronger retained and should respond stronger to an increase of the competitor (water or MeOH) [4]. This was indeed found in the AQ-HILIC mode [12] whereas under NA-HILIC conditions some deviations occurred. Aspects like solute – solvent interactions or enthalpic/entropic factors are not considered by such a simplified interpretation. Thus, the found dependence of retention on the amount of the protic modifier can neither substantiate a purely adsorption-driven retention model nor disprove the occurrence of partitioning processes.

Furthermore, mechanistic comparisons of AQ- with NA-HILIC elution conditions may be hampered to some extent by the fact that the employed NA-HILIC mobile phases were not entirely water-free. According to residual water content data provided by the manufacturers of the chosen HPLC solvents the maximal amount of water in the various NA mobile phases was below 0.05%. The role of this little water fraction in the NA-HILIC retention process is presently unknown and therefore it is justified to speculate that residual water in the eluent accumulates over time at the sorbent surface especially in case less polar NA mobile phases are used. Experimental evidence could not be found that such processes occur or affect chromatography, *i.e.* no significant shifts in retention times were observed in repetitive analysis sequences of standard samples. Yet, it was verified that the addition of water in an amount of 0.5% v/v to NA eluents containing less polar MeOH or EtOH as modifiers decreased retention times already significantly.

In any case, a proper adjustment of the amount of water in the AQ-HILIC mode as well as the careful choice of the type and amount of protic organic modifiers in NA-HILIC eluents is central to the development of optimal separation conditions for a particular chromatographic problem.

4.3 Effects of salt concentration

A significant effect of the salt concentration in the eluent on retention properties of neutral-bonded HILIC phases was reported earlier [9, 18, 30] and also found in present study, both under AQ- and NA-HILIC conditions. Higher amounts of salt in AQ-HILIC mobile phases exerted mostly a pronounced positive effect on retention while in the NA-HILIC mode some analytes departed from this trend and showed even a remarkable decrease in retention (Fig. 13). Such peculiar effects may occur due to salt-induced changes in the structure of quasistagnant protic solvent layers and solvation shells of polar functionalities, respectively. This will lead to alterations of partitioning processes. In addition to that, dynamic electrostatic shielding of interactive moieties by salt ions (potentially including ion pairing of dissociated silanols with NH_4^+) may affect polarity properties of both stationary phase (ligand functionalities, free silanols) and analytes. Ionic interactions, *e.g.* with dissociated silanols as earlier recognised for strongly basic solutes under AQ-HILIC conditions [12, 31], will of course also be influenced by the salt load although it is noted that in the present case the observed increase in retention at higher salt concentrations is contradictory to the effect that would occur in case the selected weakly basic analytes form attractive ionic interactions with silanols.

All of these scenarios will likely have effects on solute – ligand interactions (*i.e.* adsorptive forces) but will of course also affect partitioning processes (changes in hydrophilicity of sorbent and analytes). Consequently, it

follows logics that also the particular type of protic modifier will have a differential impact on these complex mechanisms. The current data clearly indicate a complex involvement of the salt in the HILIC retention process. Again, a clear distinction between partitioning and adsorption processes to describe analyte retention seems not to be reasonable and the HILIC mechanism should be regarded as having multiple interaction characteristics.

Recent data from others indicate further that both the type of cation and anion may have a pronounced impact on HILIC retention and selectivity [32]. The degree of hydration and ion pairing ability of ionic additives will certainly influence chromatographic results but intense work with different types of salts is required to understand better the role of this mobile phase parameter. Yet, the present results already confirm that the salt concentration certainly adds to the toolbox available for adjustment of proper retention and selectivity in both AQ- and NA-HILIC elution modi.

4.4 Effects of temperature

Linear van't Hoff plots are frequently found in the AQ-HILIC mode [26] and were also observed in the present study both under AQ- and NA-HILIC conditions. Exothermic transitions of the solutes from the mobile to the stationary phase as found herein seem to be somehow characteristic for HILIC-type retention while positive transfer enthalpies of charged analytes in the AQ-HILIC mode reported by others might be the result of superimposed (or even dominant) ionic interactions with ligand functionalities or surface silanols [18, 30]. Thermodynamic interpretations of HILIC retention currently face substantial speculative characteristics considering that linearity of van't Hoff plots may also be the result of compensational effects between a temperature-dependent retention enthalpy and a change in the phase ratio [33]. If an immobilised protic solvent layer is considered to be a main part of the interactive stationary phase (partitioning model) a change in density and thickness by temperature is, however, likely to occur. Such aspects need to be studied in more detail before conclusive information on HILIC thermodynamics can be drawn.

If solute–ligand (and probably silanophilic) interactions contribute significantly to the HILIC retention process water is regarded as being a strong competitor for adsorptive interactive sites. It would then follow logics that such adsorptive interactions are strengthened in case water is substituted by less polar but protic solvents. The actually observed gain in the exothermic retention enthalpy under NA-HILIC elution conditions (maximal at minimal modifier polarity) as opposed to the AQ-HILIC mode supports such a hypothesis although it also does not rule out partitioning processes.

The presence of the sugar moiety in nucleosides led mostly to more negative retention enthalphies compared to the corresponding nucleobase whereby the $\Delta\Delta H$ values of some nucleoside/nucleobase pairs decreased with decreased polarity of the protic modifier (note that such $\Delta\Delta H$ values eliminate potential contributions of temperature-dependent changes in the phase ratio as long as both compounds have access to the same stationary phase volume [33]).

In case of adsorptive interactions retention may also be less a matter of absolute analyte polarity (represented by the log D value) but more a function of the presence of specific strong adsorptive interactive sites in the molecule. Although log D values may possess some relation to the retention trend observed under AQ-HILIC conditions [6, 34] our current AQ-HILIC data lacked significant correlation of log k with calculated log D values. Retention prediction ability of this simple molecular descriptor may thus strongly depend on the studied types of packings and analytes, respectively. Instead retention modelling by multiple linear regression and artificial neural networks as recently reported in a series of papers by Jinno and coworkers [8–11, 13–15] may give access to solute descriptors that are more adequate to describe and predict the obviously complex HILIC-type retention process.

Plots of ln α versus $1/T$ revealed that the impact of temperature on chromatographic selectivity did not follow a uniform trend (Fig. 15). Depending on the types of analytes and the chosen modifier a change in temperature affected α values in a positive or negative manner. Thereby, chromatographic results obtained with mobile phases containing either water or $Et(OH)_2$ on the one hand as well as eluents containing MeOH or EtOH on the other hand were often different regarding their susceptibility towards a change in column compartment temperature. This clearly points towards mechanistic differences in the overall retention process generated by these diverse modifiers such as differential thermodynamic compensation effects of adsorptive and partition related interactions of individual analytes. With respect to method development these findings again demonstrate how different a single elution variable can affect HILIC selectivity and can thus be employed to optimise separations.

5 Concluding remarks

The presumed importance of adsorptive interactions in the overall HILIC retention process – at least for our packings and the chosen set of analytes and elution conditions – makes the careful choice of the stationary phase an important parameter in the course of method development. Particularly, it was revealed that besides

common hydroxy-groups modified silicas, sulphoxide-containing packings possess favourable properties for the separation of polar solutes in AQ- and NA-HILIC mode.

Furthermore, all of the four elution parameters investigated in the present study, *viz*. (i) the type of protic modifier, (ii) the amount of protic modifier, (iii) the amount of salt and (iv) the temperature were effective to the adjustment of retention and selectivity. Additional optimisation potential of the mobile phase likely resides in the type of salt and, in case of charged solutes and/or ligands, the pH value of the eluent. Contradictory to earlier conclusions [35, 36], we believe that particularly the type of protic modifier is an attractive yet scarcely explored mobile phase parameter which can conveniently be used to adjust chromatographic selectivity for polar probes in a dedicated manner. Nevertheless, practical aspects of the NA-HILIC mode such as the proper choice of the injection solvents and the ability for fast gradient elution (prolonged column equilibration times in case of weaker eluting eluents) need to be carefully studied before a real-world potential of NA separation conditions can be fully estimated. This applies also to the detailed evaluation of peak efficiencies and the testing of other types of polar packings, which both was beyond the scope of this initial work.

The evaluation of retention and selectivity data in both AQ- and NA-HILIC modi provided, in conjunction with intercolumn comparisons, valuable insights in the mechanisms responsible for molecular distinction in the field of HILIC-type separations. In analogy to RP chromatography [37] a plethora of adsorptive and partitioning mechanisms seem to contribute to the 'global' HILIC retention process. This calls for the development and implementation of multi-parametric models such as linear solvation energy relationships [38] to describe, understand and control these multiple interactions in a better way.

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ADDENDUM # 3

Publication (P3)

Michael Lämmerhofer
Martin Richter
Junyan Wu
Raquel Nogueira
Wolfgang Bicker
Wolfgang Lindner

Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Vienna, Austria

Original Paper

Mixed-mode ion-exchangers and their comparative chromatographic characterization in reversed-phase and hydrophilic interaction chromatography elution modes

A set of particulate silica-supported mixed-mode RP/weak anion-exchangers (RP/WAX) (obtained by bonding of *N*-undecenoylated 3-aminoquinuclidine, 3-aminotropane and 2-dimethylaminoethylamine as well as of *N*-butenoyl-(2*S*,4*S*,5*R*)-2-aminomethyl-5-[(2-octylthio)ethyl]-quinuclidine to thiol-modified silica) were chromatographically characterized in comparison to selected commercially available columns using two distinct isocratic elution modes, viz. an aqueous-rich RP-type elution mode (with 40% ACN and 60% buffer) as well as an organic solvent-rich hydrophilic interaction chromatography (HILIC)-type elution mode (95 and 90% ACN). The mixed-mode RP/WAX phases showed multimodal applicability, unlike a polar embedded RP material (Synergi Fusion RP), amino phases (Luna NH_2, BioBasic AX) or typical HILIC packings (ZIC-HILIC, TSKGel Amide-80). Principal component analysis (PCA) of the RP test data confirmed that the in-house developed RP/WAX columns as well as the Acclaim Mixed-Mode WAX-1 phase resemble each other in their chromatographic characteristics having slightly lower hydrophobic selectivity (α_{CH_2} of 1.5) than the tested Synergi Fusion RP (α_{CH_2} ~1.8). In contrast, a decrease in mixed-mode character due to lowered ion-exchange capacity and concomitantly increased RP-like behavior could be identified for other mixed-mode phases in the order of Obelisc R > Primesep B2 > Uptisphere MM3. PCA on HILIC data revealed that the RP/WAX phases behave dissimilar to TSKGel Amide-80, ZIC-HILIC and polysulfoethyl A under the chosen elution conditions. Hence, they may be regarded as complementary to these commercial stationary phases with applicability profiles for hydrophilic but also hydrophobic solutes.

Keywords: HILIC / Mixed-mode chromatography / Mixed-mode stationary phases / Reversed-phase/weak anion-exchanger / Stationary phase

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1 Introduction

RPLC, which covers a wide lipophilicity range due to its exceptionally broad applicability for organic molecules, its high efficiency and compatibility with ESI-MS, has become the method of first choice for the chromatographic separation of organic low molecular mass compounds such as pharmaceuticals, peptides, pesticides, food constituents and additives. Silica-based stationary phases with C8 or C18 bonding chemistries are typically employed as separation media for this type of LC due to their usually better peak shape for small molecules compared to organic polymer-based counterparts. These silica-based RP materials have undergone an evolution of dedicated performance optimization over decades to reinforce a single type of interaction namely hydrophobic interaction and to minimize, as far as possible, other interactions such as hydrogen bonding and cation-

Correspondence: Dr. Michael Lämmerhofer, Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringer Strasse 38, A-1090 Vienna, Austria
E-mail: michael.laemmerhofer@univie.ac.at
Fax: +43-1-3151826

Abbreviations: AQ, 3-α-aminoquinuclidine; **AT,** 3-α-aminotropane; **BAMQO,** *N*-butanoyl-(2*S*,4*S*,5*R*)-2-aminomethyl-5-[(2-octylthio)ethyl]-quinuclidine; **BuB,** butylbenzene; **DETP,** *O*,*O*-diethyl thiophosphate; **DMAE,** *N*,*N*-dimethyl-1,2-ethanediamine; **HILIC,** hydrophilic interaction chromatography; **MM,** mixed-mode; **PeB,** pentylbenzene; **RP-PEC,** RP-polar end-capped; **RP-PCA,** RP-principal component analysis; **RP/WAX,** RP/weak anion-exchanger

exchange with residual silanols. These hydrophilic secondary interactions are usually regarded as detrimental, yet are a source for proprietary selectivity profiles of the numerous commercial RP columns [1]. Limitations of conventional RP materials (type B-RP phases) for the analysis of both polar cationic and anionic compounds has led to the development of polar embedded and polar endcapped RP phases which may show better retention characteristics for polar solutes and are applicable with low or without organic modifier eluents. While they indeed expand the applicability range to more polar solutes, for very hydrophilic compounds, they may still fail. Hence, there is a need for other separation modes and this is the domain of hydrophilic interaction chromatography (HILIC) [2–7] or ion-exchange chromatography if the solutes are ionic.

Another concept that may also show an extended application range is mixed-mode chromatography [8–18]. It is by far not a brand new concept [8, 9, 19] and well-known for SPE. However, analytical chemists became only recently more aware that mixed-mode chromatographic stationary phases featuring multiple functionalities for exploitation of more than a single type of solute–sorbent interaction may be a valuable alternative or complement to RP, ion-exchange, and HILIC phases in a variety of HPLC applications (*e.g.*, exogenous and endogenous metabolite analysis [20–24], analytical and preparative peptide separations [10, 14–16], and protein purification [25–28]). Amongst the preferred application areas are the separations when RP lacks retentivity due to limited solute lipophilicity. Furthermore, the increasing necessity of complementary analysis methods for validation of RPLC assay specificity as required by regulatory authorities especially in pharmaceutical impurity profiling and stability indicating assays has driven the quest to use new sorbents with complementary selectivity profiles. Moreover, researchers are nowadays facing the problem that ever more complex samples must be analyzed comprehensively. To be able to do so successfully, multidimensional separation and analysis methods are required. A necessary precondition for the effectiveness of multidimensional LC assays is orthogonality and complementarity, respectively, in terms of retention and selectivity patterns of the distinct integrated chromatographic dimensions, and eluent compatibility of the two dimensions helps to straightforwardly realize 2-D assays technically. In view of these arguments and demands, mixed-mode phases may be a valuable complement to the more common column sets already established for a longer time.

Mixed-mode columns may appear in a variety of facets as accomplished through distinct combinations of interactive principles and altered geometrical and spatial arrangements of functional groups. The most effective seem to be, however, the combination of orthogonal,

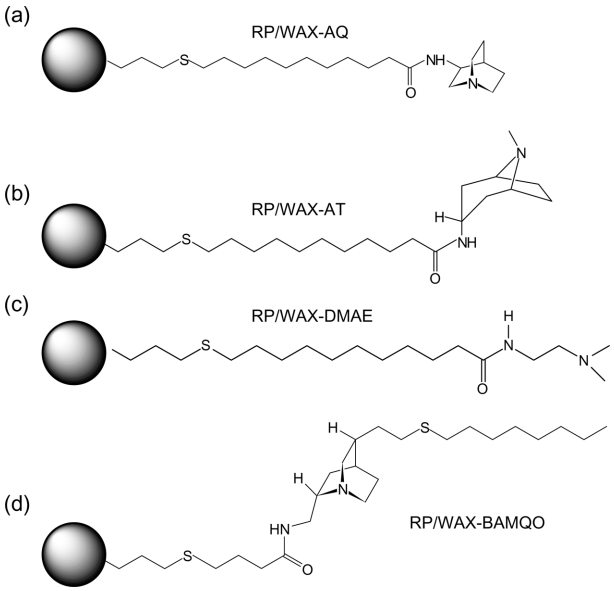

Figure 1. Mixed-mode RP/WAX phases developed in-house based on (a) *N*-undecanoyl-3-aminoquinuclidine, (b) *N*-(10-undecanoyl)-3-aminotropane, (c) *N,N*-dimethyl-*N'*-10-undecanoyl-1,2-ethanediamine, (d) *N*-butanoyl-(2*S*,4*S*,5*R*)-2-aminomethyl-5-[(2-octylthio)ethyl]-quinuclidine (BAMQO). (see also Table 1).

hydrophobic, and ionic interaction principles such as mixed-mode RP/ion-exchangers [8, 10, 13, 29, 30]. Such stationary phase materials may distinguish analytes by lipophilicity and charge differences. Besides complementary selectivity profiles, expanded application spectra, multimodal applicability (*vide infra*), and enormous flexibility for retention and selectivity tuning during method development may be named as other advantageous features. A number of such mixed-mode ion-exchangers have become commercially available from distinct suppliers recently (*vide infra*).

In the present paper, we focus on the comparative chromatographic characterization of mixed-mode anion exchangers (Fig. 1) that have been developed and synthesized in our laboratory. Some of the mixed-mode phases of Fig. 1 have been proposed recently in various applications [10, 11, 20–23] such as peptide separations [10], organophosphate metabolism studies [20], ethanol consumption marker analysis in urine [21], and chromatographic profiling with mycotoxins [22]. It is the goal of the present study, to further illustrate the chromatographic characteristics of these mixed-mode RP/weak anion-exchangers (RP/WAX) stationary phases by simple chromatographic tests providing information on their hydrophobic selectivity and ion-exchange capacity using an RP-type elution mode (40% ACN in buffer). Further, the same mixed-mode anion exchangers were evaluated in the HILIC mode by injection of nucleosides, vitamins, and xanthins as test mixtures. By these chromatographic

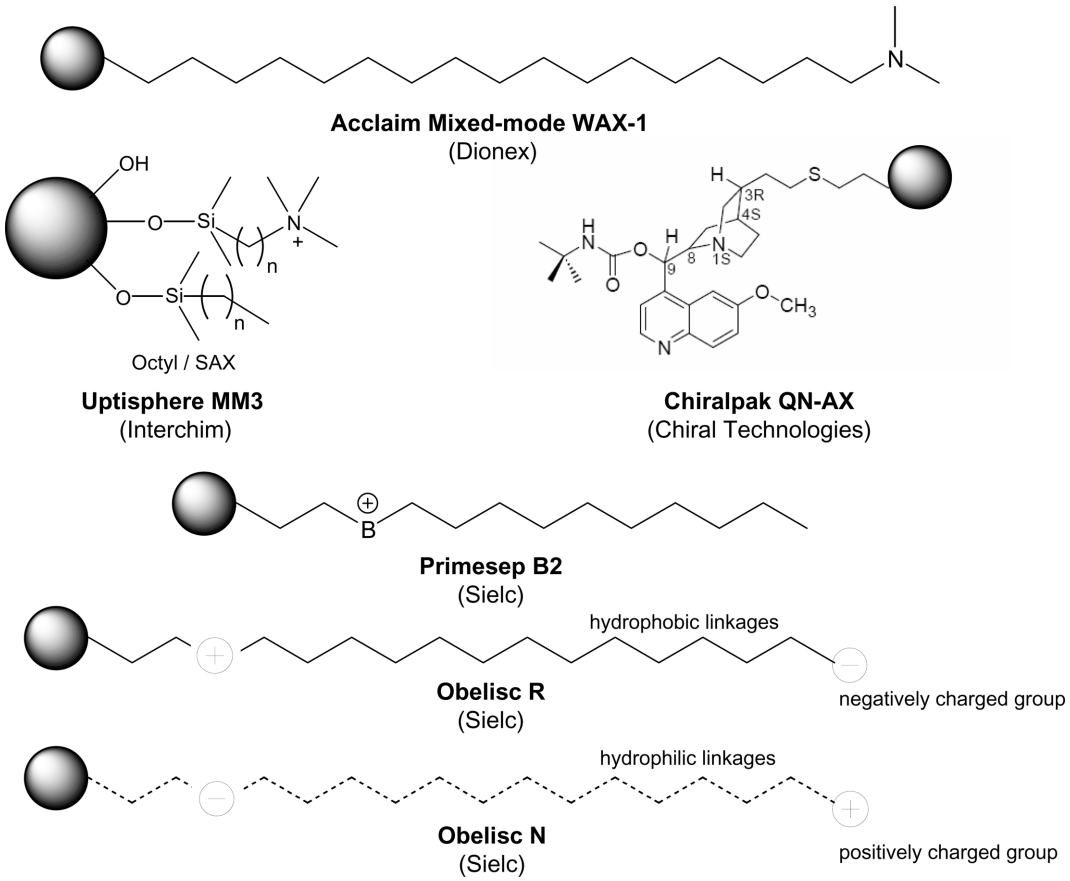

Figure 2. Tested commercially available mixed-mode phases. (Exact chemistry of stationary phases is undisclosed. Therefore the structures are given here as proposed by the suppliers).

tests similarities, dissimilarities, complementarities, and peculiarities of the RP/WAX materials under the present focus should be outlined in comparison to commercially available mixed-mode analogs (Fig. 2) as well as polar RP, amino, and HILIC phases (Fig. 3). Tests in RP and HILIC elution modes should further demonstrate the multimodal applicability of the developed RP/WAX phases.

2 Experimental

2.1 Materials

2.1.1 Stationary phases and columns

The synthesis of the in-house developed mixed-mode RP/WAX stationary phases (Fig. 1) is described elsewhere [31].

Synergi Fusion RP (Phenomenex, Torrance, CA, USA) is a silica-based polar embedded C18 phase (nominal pore size, 80 Å). Luna NH_2 (from Phenomenex) is, according to the supplier, an endcapped amino phase (pore size, 100 Å). BioBasic AX (from Thermo Scientific, Waltham, MA, USA) is a polyethyleneimine (PEI) polymer-coated weak anion exchanger (300 Å pore size). Acclaim Mixed-Mode WAX-1 (Dionex, Sunnyvale, CA, USA) is a silica-based mixed-mode stationary phase that incorporates both hydrophobic and weak anion-exchange properties (pore size, 120 Å). The Uptisphere MM3 (from Interchim, Montluçon, France) is an octyl/strong anion-exchange mixed-mode stationary phase (pore size, 120 Å). Primesep B2 (from SIELC Technologies, Prospect Heights, IL, USA) is a weakly basic mixed-mode phase which incorporates, according to specifications of the supplier, also carboxylic acid functional groups that are, however, not ionized at mobile phase pH below 5. Hence, at low pH the Primesep B2 column behaves like a mixed-mode RP/weak anion-exchanger. Obelisc R and Obelisc N, also from SIELC Technologies, are zwitterionic type mixed-mode phases with either RP or normal-phase characteristics (both with 100 Å pore size). CHIRALPAK QN-AX (Chiral Technologies Europe, Illkirch, France) is a weak anion-exchange type chiral stationary phase (120 Å pore size). TSKGel Amide-80 (Tosoh Bioscience, Stuttgart, Germany) is a spherical silica-based HILIC-type stationary phase with covalently bonded carbamoyl groups (80 Å nominal pore size; the pore size of the base silica is 100 Å). Polysulfoethyl A (PolyLC, Columbia, MD, USA) is a hydrophilic

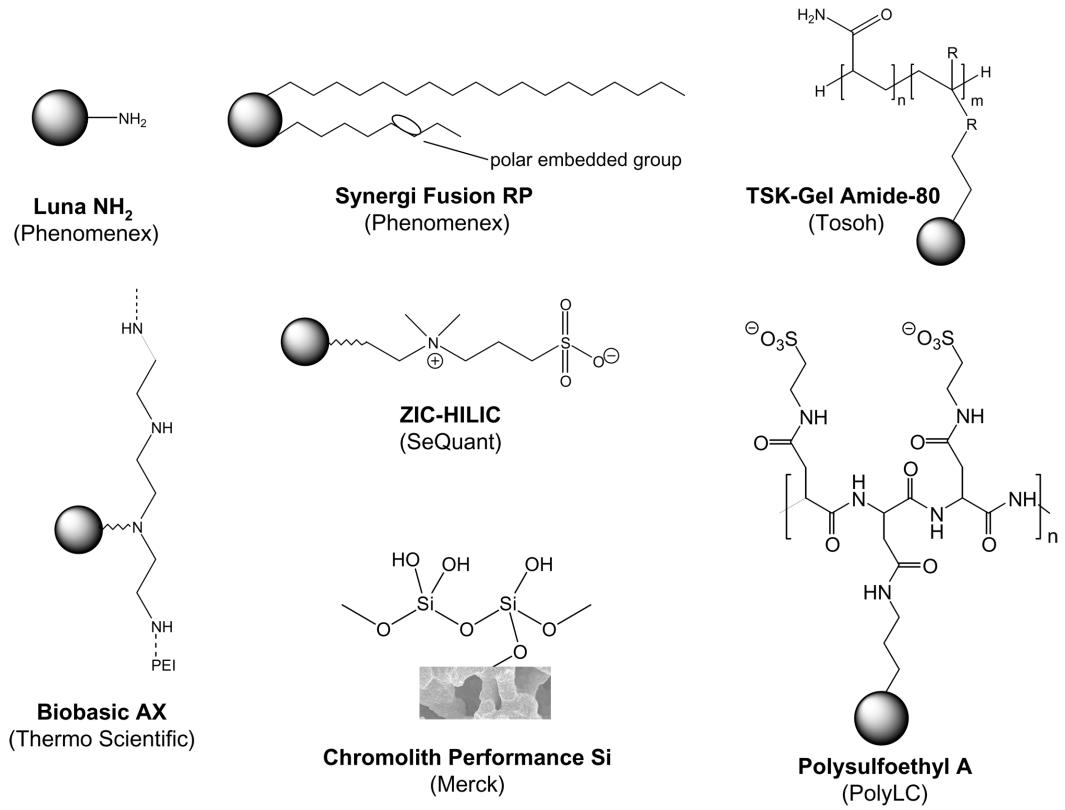

Figure 3. Tested commercially available amino, polar RP, and HILIC columns. (Exact chemistry of stationary phases is undisclosed, therefore the structures are given here as proposed by the suppliers).

sulfoethyl-group bearing polyaspartamide-based strong cation-exchanger (300 Å pore size). ZIC-HILIC (SeQuant, Umea, Sweden) is a silica-based sulfobetaine-type bonded phase which is particularly suitable for HILIC separations (200 Å pore size). Chromolith Performance Si 60 (Merck, Darmstadt, Germany) is a native PEEK-cladded monolithic silica column (100 × 4.6 mm id) with a macropore diameter of 2 μm and a mesopore diameter of 13 nm.

Some characteristic properties of all the tested phases are summarized in Table 1. Several of the commercial columns have been emptied and were repacked into 100 × 4 mm id columns. Unless otherwise stated the column dimension was 100 × 4 mm id and the particle diameter was 5 μm, except Synergi Fusion RP for which particle size was 4 μm.

2.1.2 Synthesis of 3-(*N,N*-dimethylamino)propyl-silica type weak anion-exchanger (WAX)

Kromasil 100, 5 μm (3.0 g) (EKA, Bohus, Sweden), (*N,N*-dimethylaminopropyl)trimethoxysilane (380 μL) (ABCR, Karlsruhe, Germany), and 4-dimethylaminopyridine (10 mg) (Sigma-Aldrich, Vienna, Austria) were refluxed in distilled toluene (50 mL) for 20 h under continuous stream of nitrogen and stirring. The modified silica gel was washed several times with chloroform, methanol, and diethylether. Remaining silanol groups were capped with *N,O*-bis(trimethylsilyl)acetamide (1.7 mL) (Aldrich) by refluxing the modified sorbent in toluene for 7 h. The modified silica gel was washed with toluene, chloroform, and methanol, dried for 72 h, and passed through a 0.0025 mm sieve. The calculated selector coverage was 0.40 mmol/g as determined by elemental analysis (3.45% C, 0.90% H, 0.81% N). The end-capping resulted in carbon increase (4.59% C, 1.11% H, 0.75% N).

2.1.3 Chemicals

Most of the test compounds (butylbenzene, pentylbenzene, caffeine, theobromine, theophylline, adenosine, cytidine, guanosine, thymidine, uridine, ascorbic acid, nicotinic acid, pyridoxine, riboflavin, thiamine) were supplied by Sigma-Aldrich. *O,O*-Diethyl thiophosphate (DETP) was prepared by hydrolyzation of *O,O*-diethyl chlorothiophosphate (from Aldrich) in a mixture of ACN/water (3:1, v/v) in the presence of an equimolar amount of triethylamine. *N-tert*-Butoxycarbonyl-prolyl-phenylalanine (Boc-Pro-Phe) was purchased from Bachem (Buchs, Switzerland).

Mobile phases were prepared from glacial acetic acid (Merck; purchased from VWR International, Vienna, Aus-

Table 1. Stationary phases and columns, respectively, used for the comparative column characterization

	Type	%C	%H	%N	Surface Area [m^2/g]	Sel. amt.[b] [mmol/g]
In-house developed RP/WAX columns						
N-undecanoyl-3-aminoquinuclidine based (AQ240) (low capacity) (Fig. 1a)	MM	14.86	2.22	0.67	300	0.24
N-undecanoyl-3-aminoquinuclidine based (AQ360) (high capacity) (Fig. 1a)	MM	17.12	2.83	1.00	300	0.36
N-(10-undecanoyl)-3-α-aminotropane based (AT) (Fig. 1b)	MM	18.28	2.78	1.17	300	0.42
N,*N*-dimethyl-*N*′-10-undecanoyl-1,2-ethanediamine based (DMAE) (Fig. 1c)	MM	12.62	2.21	1.27	300	0.42
N-Butanoyl-(2*S*,4*S*,5*R*)-2-aminomethyl-5-[(2-octylthio)-ethyl]quinuclidine (BAMQO) (Fig. 1d)	MM	13.99	2.35	1.20	300	0.36
Commercial columns/comparison columns						
Acclaim Mixed Mode WAX-1 (Dionex)	MM	12.70	2.50	1.46	300	0.52 (2N)[a]
Uptisphere MM3 (Interchim)	MM	8.14	1.79	0.19	320	0.14 (1N)[a]
Primesep B2 (Sielc)	MM	8.38	1.83	0.65	n/a	0.46 (1N)[a]
Obelisc R (Sielc)	MM	8.93	1.92	0.66	n/a	0.47 (1N)[a]
Obelisc N (Sielc)	MM	4.59	1.21	0.30	n/a	0.21 (1N)[a]
Chiralpak QN-AX (Chiral Technologies)	Chiral WAX	15.14	2.38	1.59	300	0.38
Luna NH_2 (Phenomenex)	Amino	10.09	2.31	2.84	400	2.32
BioBasic AX (Thermo Scientific)	Amino	3.7	0.77	0.58	100	0.22
3-(*N*,*N*-dimethylamino)propyl-silica (WAX) (in-house)	WAX	4.59	1.11	0.75	300	0.40
Synergi fusion RP (Phenomenex)	RP-PEC	12.00	n/a	–	475	n/a
TSKGel Amide-80 (Tosoh)	HILIC	11.06	1.99	2.46	n/a	1.76 (1N)[a]
Polysulfoethyl A (PolyLC)	HILIC/SCX	9.95	1.74	3.47	100	0.55 (1S)[a]
ZIC-HILIC (SeQuant)	HILIC	8.74	1.77	1.02	140	0.59 (1S)[a]
Chromolith performance Si (Merck)	Silica	–	–	–	300	–

[a] Exact surface chemistry undisclosed. Element used for calculations and its assumed number in the ligand in parenthesis.
[b] Sel. amt = Selector amount.

tria), analytical grade ammonia (Merck), HPLC grade water (double distilled) and HPLC grade ACN (Merck).

2.2 Instrumentation

The chromatographic experiments were carried out on an Agilent (Waldbronn, Germany) 1050 Liquid Chromatograph consisting of a single channel pump, an autosampler, an Agilent 1200 variable wavelength detector, and a column thermostat of W.O. Electronics (Langenzersdorf, Austria). The data were processed by the Agilent Chemstation software.

The pH of the mobile phase ($_{w}^{s}$pH) was measured in the hydro-organic mobile phase mixtures with a pH 540 GLP pH-meter from WTW (Weilheim, Germany).

2.3 RP test conditions

The mobile phase for the RP-HPLC test run was composed of a mixture of ACN and water (40:60, v/v) containing in total 0.29% v/v glacial acetic acid (C_{tot} = 50 mM). The pH of the mixture was adjusted to pH 6 with ammonia. The flow rate was adjusted to an equivalent linear flow velocity u of 1.7 mm/s. The test mixture consisted of butylbenzene, pentylbenzene, DETP, and Boc-Pro-Phe (dissolved in the mobile phase each at a concentration of 0.8 mg/mL). Acetone was employed as void volume marker. The injection volume of the sample was 10 µL. The column was thermostated at 25°C and the detection wavelength was set to 220 nm.

2.4 HILIC test conditions

The mobile phase for the HILIC test mixture was composed of ACN/ammonium acetate buffer (95:5 v/v) for the xanthins test and (90:10 v/v) for the nucleosides and vitamins tests, respectively, and the total buffer concentration was 5 mM. The eluent pH was unadjusted and had an apparent pH ($_{w}^{s}$pH) of around 8 when measured with a glass electrode in the hydroorganic mixture and use of aqueous calibration buffers [32, 33]. The flow rate was adjusted to the same linear velocity (u = 1.7 mm/s). The test mixtures are specified later and the concentration was about 1 mg/mL for each compound dissolved in the mobile phase. The injection volume of the sample was 10 µL. The column was thermostated at 25°C and the detection wavelength was set to 220 nm. Toluene was used as the void marker.

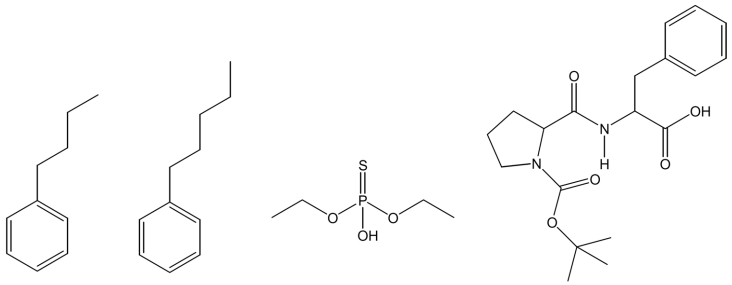

Figure 4. Structures as well as log *P* and log *D* values (at pH 6), respectively, for the RP test compounds.

3 Results and discussion

3.1 Comparative column characterization by isocratic RPLC elution mode

3.1.1 Comparison of various mixed-mode RP/WAX phases

A series of structural analogs of RP/WAX phases (Fig. 1) have been synthesized and were comparatively evaluated by an RPLC test consisting of hydrophobic butylbenzene (BuB, **1**) (log *P* = 4.27), pentylbenzene (PeB, **2**) (log *P* = 4.80), DETP, **3** (a polar acidic compound with log *D* at pH 6 of −2.8), and Boc-Pro-Phe (**4**) (a more lipophilic acidic compound with log $D_{6.0}$ of −0.6). The chromatographic results are given in Table 2 and Fig. 5. Some characteristics of the stationary phases are summarized in Table 1.

Structurally, these mixed-mode RP/WAX phases are assembled modularly from a number of distinct functional groups representing diverse interactive sites which form the basis for the peculiarity and multimodal applicability of these adsorbents. The chemical surface modification of the materials of Figs. 1a–c is composed of a hydrophobic alkyl strand with polar embedded groups (sulfide and amide functionalities) and a terminating tertiary amino group as the weak anion-exchange site. The WAX moiety in these three phases differs in the type of the incorporated amino groups being derived from 3-aminoquinuclidine (RP/WAX-AQ; Fig. 1a), 3-*α*-aminotropane (RP/WAX-AT; Fig. 1b), and 2-(*N*,*N*-dimethylamino)ethane (RP/WAX-DMAE; Fig. 1c), respectively. The RP/WAX phase of Fig. 1d deviates from these general structural features in that it has shifted the WAX-site to the interior of the lipid layer and has an additional sulfide linking group. The ligand loadings have been adjusted to yield a comparable total ion-exchange capacity (∼0.39 ± 0.03 mmol/g), except for RP/WAX-AQ240 that was intentionally synthesized with lower RP/WAX selector density (∼240 μmol/g) to show this effect. Under the test conditions (pH 6), the WAX site is fully protonated in case of all the presented ligands.

The results of the current chromatographic RP test (Table 1 and Fig. 5) clearly reveal similar retention patterns for the distinct RP/WAX selectors and confirm their mixed-mode retention mechanism. Regardless of the ligand structure, all the tested materials showed a sufficient hydrophobicity and anion-exchange capacity thus being capable of retaining both the neutral hydrophobic alkylbenzenes and the more lipophilic acid as well as the polar acid likewise. In any case, the surfaces of these RP/WAX materials are still hydrophobic enough to enable a baseline resolution of neutral compounds that just differ in a single methylene increment (*vide infra*). On the other hand, the superimposed anion-exchange retention mechanism expands the applicability profile to polar acids like DETP for which a sufficient retentivity can be obtained. Such compounds usually elute in the polar bulk close to the void time on RP columns, even with strongly acidic eluents. It is also worth pointing out the specific elution order between the hydrophilic and hydrophobic acids which simply correlates with their lipophilicities (see Fig. 4) and is the same on all the tested RP/WAX phases (Fig. 5). In contrast, the position of the acidic solutes relative to the neutral compounds in the chromatogram can be easily varied or adjusted by either a change of the total ion-exchange capacity of the stationary phase through alteration of the ligand coverage or by a change of the actual ion-exchange capacity through variations of the ionic strength or the pH of the eluent [22]. With a slight reduction of the anion-exchange capacity of the mixed-mode phase from 0.36 to 0.24 mmol/g the retentivity of the RP/WAX-AQ phase for the acidic constituents in the test mix was diminished, while both the hydrophobic selectivity between the alkylbenzenes and the acid selectivity between hydrophilic DETP and more lipophilic Boc-Pro-Phe were still more or less maintained (*cf.* Figs. 5a and b).

It can be concluded from these chromatographic test results and earlier investigations [22] that the RP/WAX phases with aminoquinuclidine and aminotropane selectors of around 360 μmol/g are more or less equivalent. Yet, the RP/WAX-AQ360 phase emerged as the first choice

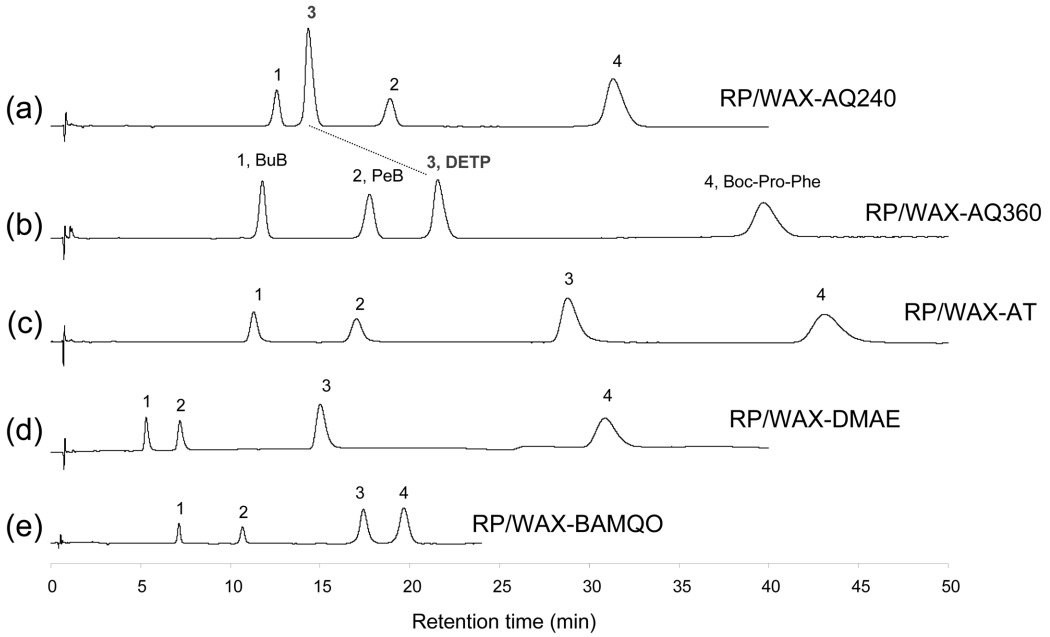

Figure 5. Comparison of chromatograms obtained with various in-house developed RP/WAX phases employing RP-elution mode. Stationary phases based on (a) *N*-undecanoyl-3-aminoquinuclidine (240 μmol/g), (b) *N*-undecanoyl-3-aminoquinuclidine (360 μmol/g), (c) *N*-(10-undecanoyl)-3-α-aminotropane, (d) *N*,*N*-dimethyl-*N*′-10-undecanoyl-1,2-ethanediamine, (e) BAMQO. Conditions see Section 2. Solutes: 1, butylbenzene; 2, pentylbenzene; 3, DETP; 4, Boc-Pro-Phe.

material and is therefore used in the following tests for further comparisons with commercial phases.

3.1.2 Comparison of mixed-mode RP/WAX with amino and RP phases

The above data and earlier applications clearly revealed that the stationary phases of Fig. 1 display a mixed-mode retention mechanism which may be *inter alia* utilized for a flexible retention and selectivity adjustment. This peculiarity of such materials in terms of retention characteristics should be illustrated at this place by comparison with the corresponding closest art parental single-mode stationary phases, namely an RP phase and two different amino phases (Fig. 6).

Synergi Fusion RP has been selected for this purpose as the RP column. According to the supplier, it contains silica with octadecyl-strands and a second shorter alkyl ligand with a polar embedded group. Whatsoever, it should exhibit enhanced retentivity for polar acids as compared to classical C18 phases. As can be seen from the chromatograms of Fig. 6b with the selected conditions (40% ACN and 60% buffer pH 6) only the more lipophilic acid Boc-Pro-Phe (**4**) can be retained while the polar acid DETP (**3**) is eluted with the solvent front. It is obvious that the RP/WAX phase shows greatly improved retentivity and selectivity due to overlapped anion-exchange processes and hydrophobic interaction contributions. The alkylbenzenes are stronger retained than the acids which is the expected elution order on basis of the lipophilicity of the test solutes. The polar embedded groups have, if any, only little effect on the retention of the acidic solutes at pH 6. Concomitantly, the Synergi Fusion RP phase exhibits slightly enhanced hydrophobic selectivity as measured by the methylene selectivity (α_{CH_2} = 1.8) than the RP/WAX-AQ360 phase (α_{CH_2} = 1.5). Nevertheless, this hydrophobic selectivity of the RP/WAX phase is still sufficient in many instances for separation of solutes merely based on lipophilicity differences.

Amino phases such as Luna NH_2 or BioBasic AX may be regarded as the parental charged counterparts of RP/WAX phases. They should have the potential to retain and separate acidic solutes by anion-exchange processes as well, and this is confirmed by the chromatograms in (Figs. 6c and d). In this context it is, however, striking that the elution order is reversed on these polar amino phases as compared to the lipophilic RP/WAX phase in that the more lipophilic Boc-Pro-Phe (**4**) is eluted prior to DETP (**3**). Along with a complete lack of retentivity for the neutral alkylbenzenes (**1** and **2**) and hence of hydrophobic selectivity this is the most characteristic distinctive feature between RP/WAX and classical amino phases. To account for the fact that the present mixed-mode RP/WAX phases are all tertiary amine phases while classical amino phases are primary or secondary amines, we synthesized 3-(*N*,*N*-dimethylamino)propyl-silica as a WAX phase with a tertiary amine for sake of comparison. As can be seen from Fig. 6e it shows only little selectivity for the polar and lipophilic acid (with

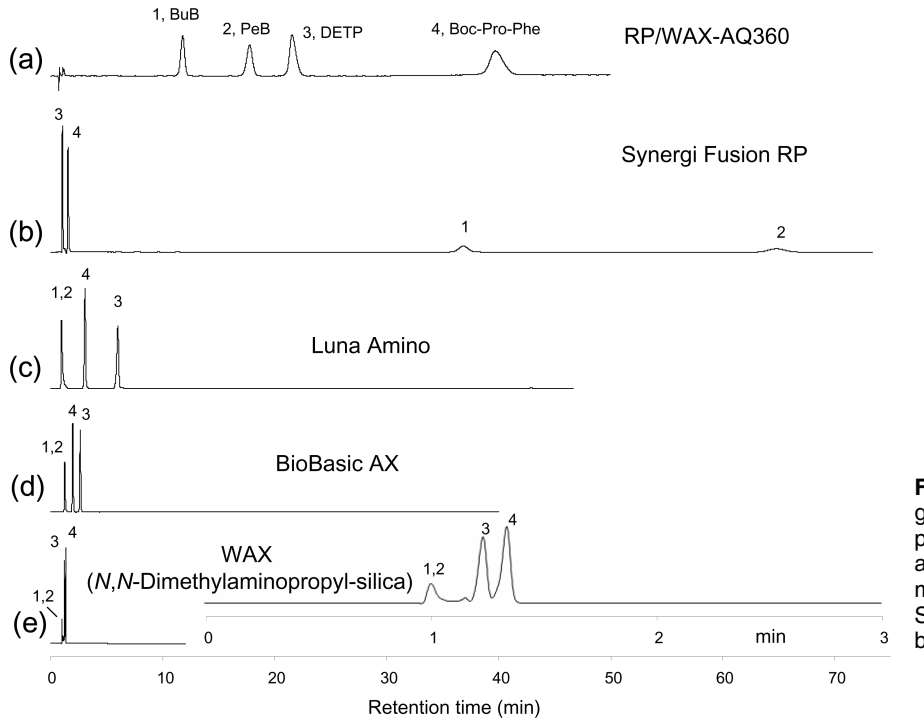

Figure 6. Comparison of chromatograms obtained with (a) RP/WAX, (b) polar RP phase and (c–e) various amino phases employing RP-elution mode. For conditions, see Section 2. Solutes: **1**, butylbenzene; **2**, pentylbenzene; **3**, DETP; **4**, Boc-Pro-Phe.

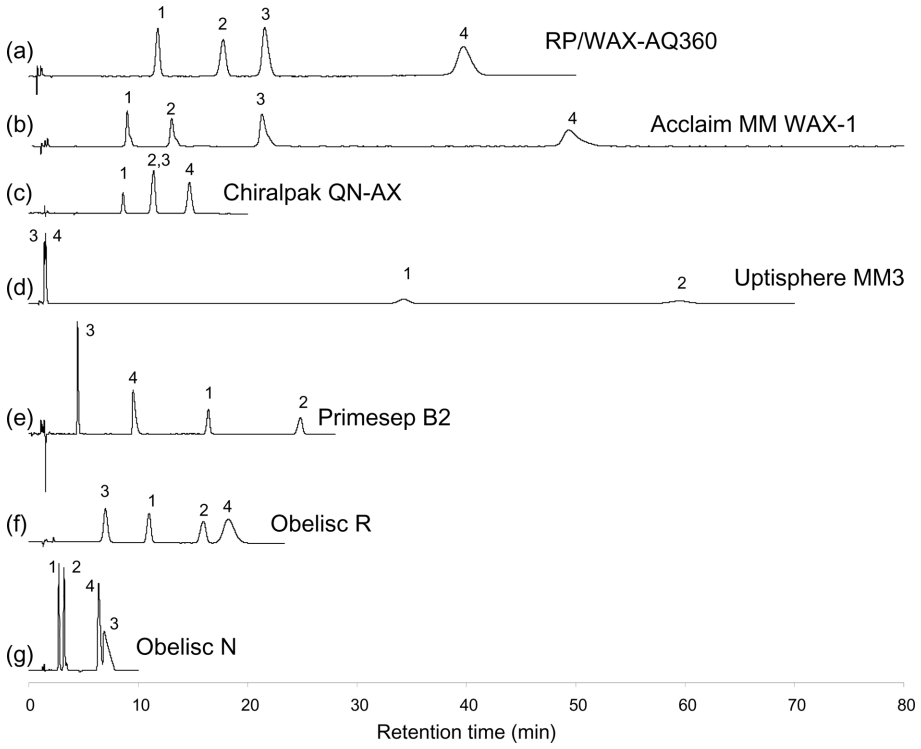

Figure 7. Comparison of the separation of the RP-test mixture on in-house developed RP/WAX-AQ360 phase with those obtained on commercially available mixed-mode phases employing RP-elution mode. Conditions see Section 2. Solutes: **1**, butylbenzene; **2**, pentylbenzene; **3**, DETP; **4**, Boc-Pro-Phe.

same elution order as the RP/WAX phase) while the alkylbenzenes are eluted unseparated with the void volume. Obviously, the surface lipophilicity of the WAX phase is, like for classical amino phases, too low to give rise to hydrophobic selectivity contributions. Hence, the improved retention and selectivity profiles of the present RP/WAX phases are due to their specific surface chemistries with their dedicated multiple, yet complementary interaction sites.

3.1.3 Comparison with commercial mixed-mode phases

The RP-test mix has also been run on a set of commercially available mixed-mode phases (see Fig. 7 and Table 2

Table 2. Chromatographic results for the RP test (for structures and log *P*/log $D_{6.0}$ see Fig. 3)[a)]

	k_{BuB}	k_{PeB}	k_{DETP}	$k_{Boc\text{-}Pro\text{-}Phe}$	α_{CH_2}
In-house developed RP/WAX columns					
RP/WAX-AQ240 (low capacity) (Fig. 1a)	11.01	17.03	12.69	28.89	1.55
RP/WAX-AQ360 (high capacity) (Fig. 1a)	11.80	18.36	25.71	45.91	1.56
RP/WAX-AT (Fig. 1b)	11.46	17.78	30.75	46.51	1.55
RP/WAX-DMAE (Fig. 1c)	4.43	6.35	14.35	30.57	1.43
RP/WAX-BAMQO (Fig. 1d)	10.36	15.99	26.68	30.27	1.54
Commercial/comparison columns					
Acclaim Mixed Mode WAX-1 (Dionex)	5.52	8.47	14.45	34.75	1.53
Uptisphere 5 MM3 (Interchim)	27.73	48.89	0.19	0.28	1.76
Primesep B2 (Sielc)	9.97	15.59	1.97	5.36	1.56
Obelisc R (Sielc)	6.22	9.47	3.59	10.97	1.52
Obelisc N (Sielc)[b)]	1.00	1.35	4.00	3.64	1.35
Chiralpak QN-AX (Chiral Technologies)	4.11	5.76	5.76	7.69	1.40
Luna NH_2 (Phenomenex)	With t_0	With t_0	4.39	1.76	–
BioBasic AX (Thermo Scientific)	With t_0	With t_0	1.05	0.53	–
WAX (in-house)	With t_0	With t_0	0.12	0.22	–
Synergi Fusion RP (Phenomenex)	24.17	43.28	With t_0	0.06	1.79
TSKGel Amide-80 (Tosoh)	With t_0	With t_0	With t_0	With t_0	–
PolysulfoethylA (PolyLC)	With t_0	With t_0	With t_0	With t_0	–
ZIC-HILIC (SeQuant)	With t_0	With t_0	0.14	With t_0	–
Chromolith performance Si (Merck)	With t_0	With t_0	With t_0	With t_0	–

[a)] Conditions see Section 2, all measurements at pH 6.0.
[b)] Same conditions but pH 4.5.

for the detailed results). Amongst the commercial mixed-mode phases (Fig. 2) the Acclaim Mixed-Mode WAX-1 [13, 30] displayed the most similar retention characteristics and selectivity profiles to the RP/WAX-AQ360 phase both with regard to hydrophobic selectivities (α_{CH_2} = 1.53 *vs.* 1.50 for RP/WAX-AQ360) and ion-exchange capacity as well, as already described previously [22] (*cf.* Figs. 7a and b). According to the supplier's information, the chromatographic ligand of Acclaim Mixed-Mode WAX-1 contains a hydrophobic alkyl chain terminated by a weak anion-exchange group which is bonded to silica. Elemental analysis data indicate that this mixed-mode phase has also a comparable yet little higher selector loading (*ca.* 0.52 mmol/g based on calculations with two nitrogens in the chromatographic ligand molecule).

All the other phases have a significantly lower actual ion-exchange capacity as evident from lower retention factors for the acidic compounds. Still, Primesep B2 (Fig. 7e) and Obelisc R (Fig. 7f) show a hydrophobic selectivity α_{CH_2} of 1.56 and 1.52, respectively, which is a typical value for mixed-mode RP/anion-exchangers (α_{CH_2} = 1.50 for RP/WAX-AQ360). Moreover, they also feature the characteristic acid selectivity profile and elution order, respectively, between DETP (**3**) and Boc-Pro-Phe (**4**) (polar acid eluted before more lipophilic acid). A more detailed interpretation is difficult, because the exact structures of the chromatographic ligands as well as the specific surface area and thus ligand density on the surface are undisclosed. However, a sketch of the stationary phase structures, taken from the products' websites, is given in Fig. 2. Primesep B2 contains a weakly basic group that is embedded in an alkyl strand. It has additionally incorporated, according to the supplier's information, weakly acidic groups which would explain the lower actual ion exchange capacity of this material. Obelisc R has a zwitterionic chromatographic ligand with an embedded cationic and a terminal anionic group connected by hydrophobic linkages. From the retention profiles it may be derived that the phase has at pH 6 excess positive charge leading to a significant yet comparably low anion-exchange capacity.

In contrast to Obelisc R, Obelisc N has hydrophilic linkages and the arrangement of the ionic groups is just reversed (terminal cationic and embedded anionic group). Accordingly, it produces a significantly lower hydrophobic selectivity than the other mixed-mode phases (α_{CH_2} = 1.35 *vs.* 1.50 for RP/WAX-AQ360) and moreover reveals, like polar amino phases, a reversed elution order for the two acids (Boc-Pro-Phe is eluted before DETP) (Fig. 7g).

Figure 7c shows also the chromatogram of the RP-test mix on a chiral stationary phase with a quinuclidine ring, Chiralpak QN-AX. While both hydrophobic selectivity as well as actual anion-exchange capacity seems to be lower despite a comparable amount of chromatographic ligand bonded to the support, its overall retention profile resembles a mixed-mode character. Furthermore, another mixed-mode phase Uptisphere MM3 has been included in the test set. This stationary phase behaves significantly different from the RP/WAX-AQ360 phase and

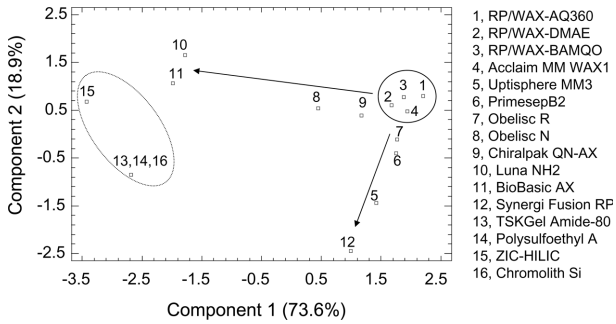

Figure 8. Results of the PCA on the RP-elution data: Score plot of the two first principal components.

the other tested stationary phases. It is striking that it has almost the same hydrophobic selectivity (α_{CH_2} = 1.76) as the Synergi Fusion RP (α_{CH_2} = 1.79). On the other hand, its anion-exchange capacity is almost not existing (hydrophilic and also hydrophobic acids elute with or close to t_0) (Fig. 7d). It seems that the quaternary ammonium ions are introduced as a second ligand into a typical RP phase by an end-capping procedure (polar end-capped phase). This speculative statement is supported by the low molar amount of nitrogen found by elemental analysis (0.14 mmol/g) (see Table 1).

Other commercially available polar phases which have been tested and are often or typically used for HILIC (like TSKGel Amide-80, Polysulfoethyl A, ZIC-HILIC, Chromolith Performance SI which is a plain silica phase), not surprisingly, did not show any retention nor selectivity (see Table 2) because the employed eluent does not constitute the recommended elution mode for these phases.

3.1.4 Principal component analysis

The generated data matrix of the RP test (retention factors and selectivities) has been processed by principal component analysis (PCA) to elucidate similarities/dissimilarities between the distinct stationary phases. As can be seen from the score plot in Fig. 8, this statistical projection procedure clearly clusters the columns of similar character, while the ones being most dissimilar are farthest away from each other in the 2-D plot of PC1 and PC2 (the values given in parenthesis on the axis description refer to the percentage of variance in the data set explained by the corresponding component and latent variable, respectively).

From this scatter plot of the first two components, it becomes obvious that the RP/WAX columns (1, 2, 3, 4) are clustered together as they show similar chromatographic properties. The arrow from 7 to 12 indicates the direction in which the mixed-mode character of the tested stationary phases decreases (Obelisc R > Primesep B2 ≫ Uptisphere MM3) and the chromatographic profiles resemble more and more those of a polar embedded group RP material (in other words a decrease in the actual anion-exchange capacity can be observed in the given order). The arrow from 9 to 11 on the other hand denotes the direction in which the mixed-mode character varies in such a way to become more and more similar to a typical amino-phase character (being more or less tantamount to a decrease in hydrophobic selecitivity). While the Chiralpak QN-AX column has still a reasonable mixed-mode character, that of Obelisc N is already significantly less as was underpinned by the chromatographic profiles above. The columns TSKGel Amide-80, Polysulfoethyl A, Chromolith Performance Si, and ZIC-HILIC which are normally propagated as typical HILIC columns showed similar behavior in the RP-test, namely inadequate retention for the test compounds under the chosen aqueous-rich elution conditions. So, PCA clearly grouped together stationary phases of similar characteristics and underlined the trends discussed above.

3.2 Comparative column characterization by HILIC tests

3.2.1 Discussion of HILIC test results

The mixed-mode RP/WAX stationary phases under study (Fig. 1) have multimodal applicability which was already outlined previously [22, 23]. Besides separations in an anion-exchange mode for acids and in RP mode, *e.g.*, for neutral lipophilic compounds, they possess applicability in HILIC mode as well, namely for polar compounds with ACN-rich aqueous-organic eluents [21 – 23]. To get more detailed insight on how the presented in-house developed RP/WAX test columns behave in comparison to classical HILIC columns and analogs on the market, we have undertaken comparative HILIC tests using three different test mixtures. One of the test mixtures contained xanthins (caffeine, theobromine, and theophylline), another one contained nucleosides (adenosine, cytidine, guanosine, thymidine, and uridine), and the last one contained water-soluble vitamins (ascorbic acid, nicotinic acid, pyridoxine, riboflavin, thiamine). All are frequently employed solutes to test HILIC columns [3]. Their structures are depicted in Fig. 9 and the chromatographic results are summarized in Tables 3 – 5.

Figure 10 illustrates the resultant chromatograms for the xanthins. It is evident that an HILIC mode separation is realized on all of the stationary phases tested and shown in Fig. 10, because without a single exception, caffeine (trimethyl-xanthine) eluted before the more polar 1,3-dimethyl xanthins (theobromine and theophylline). It is also notable that the basic columns, *i.e.*, the RP/WAX columns as well as the Luna NH_2 phase, show different elution profiles and better separations, in particular for the dimethyl xanthins. An elution order of caffeine < theophylline < theobromine was expected from the calcu-

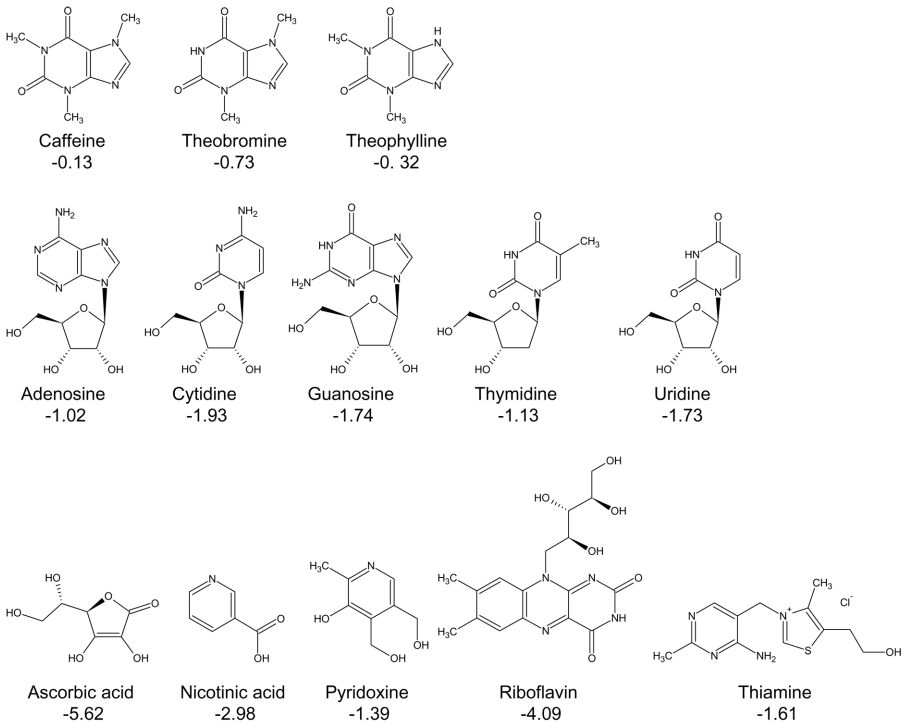

Figure 9. Structures as well as log *D* values (pH 8) for the employed HILIC test compounds.

Table 3. Chromatographic results for the HILIC test for xanthines (for structures and log *P*/log $D_{8.0}$ see Fig. 9)[a)]

	$k_{caffeine}$	$k_{theobromine}$	$k_{theophylline}$
In-house developed RP/WAX columns			
RP/WAX-AQ360 (high capacity) (Fig. 1a)	0.17	0.35	1.11
RP/WAX-DMAE (Fig. 1c)	0.10	0.28	0.76
RP/WAX-BAMQO (Fig. 1d)	0.03	0.18	0.80
Commercial/comparison columns			
Acclaim Mixed Mode WAX-1 (Dionex)	0.10	0.26	0.84
Uptisphere 5 MM3 (Interchim)	With t_0	With t_0	With t_0
Primesep B2 (Sielc)	0.02	0.02	0.11
Obelisc R (Sielc)	0.06	0.14	0.26
Obelisc N (Sielc)	0.15	0.22	0.22
Chiralpak QN-AX (Chiral Technologies)	0.10	0.24	0.44
Luna NH_2 (Phenomenex)	0.31	0.67	3.19
BioBasic AX (Thermo Scientific)	0.08	0.18	0.18
Synergi Fusion RP (Phenomenex)	With t_0	With t_0	With t_0
TSKGel Amide-80 (Tosoh)	0.40	0.76	1.16
PolysulfoethylA (PolyLC)	0.20	0.45	0.51
ZIC-HILIC (SeQuant)	0.28	0.63	0.63
Chromolith performance Si (Merck)	0.19	0.19	0.19

[a)] Conditions see Section 2.

lated log *D* values which may be a descriptor for the relative hydrophilicity of the solutes. While this order is matched on the TSKGel Amide-80 phase, this trend is puzzled on the basic RP/WAX phases and Luna NH_2. The deviating stronger retention of theophylline points toward a superimposed ionic interaction increment of the slightly more acidic theophylline ($pK_{a,theophylline}$ = 8.6) as compared to theobromine ($pK_{a,theobromine}$ = 9.9). On phases with neutral, cationic, and zwitterionic surface bonding the dimethyl xanthins elute much closer to each other and in case of TSKGel Amide-80 and polysulfoethyl A even with reversed elution order. The elution profiles on these phases resemble more the expected trends. However, electrostatic repulsion (due to residual silanols or negative charge from the sulfonic acid groups on the surface) could also be made responsible for a lower retention of theophylline on these columns. The surface hydrophilicity of Primesep B2 appears to be too

Table 4. Chromatographic results for the HILIC test for nucleosides (for structures and log *P*/log $D_{8.0}$ see Fig. 9)[a)]

	$k_{thymidine}$	$k_{adenosine}$	$k_{uridine}$	$k_{cytidine}$	$k_{guanosine}$
In-house developed RP/WAX columns					
RP/WAX-AQ360 (high capacity) (Fig. 1a)	0.79	1.59	1.89	4.12	7.52
RP/WAX-DMAE (Fig. 1c)	0.66	1.13	3.82	9.18	17.62
RP/WAX-BAMQO (Fig. 1d)	0.13	1.31	1.09	2.33	4.59
Commercial/comparison columns					
Acclaim Mixed Mode WAX-1 (Dionex)	0.88	1.67	1.90	4.18	7.15
Uptisphere 5 MM3 (Interchim)	With t_0	0.15	0.15	0.81	1.05
Primesep B2 (Sielc)	With t_0	0.10	With t_0	0.22	0.22
Obelisc R (Sielc)	0.09	0.48	0.48	2.15	2.69
Obelisc N (Sielc)	0.23	0.59	0.48	2.75	1.61
Chiralpak QN-AX (Chiral Technologies)	0.15	0.15	0.15	0.29	0.29
Luna NH_2 (Phenomenex)	1.21	3.80	3.80	11.99	19.18
BioBasic AX (Thermo Scientific)	0.35	0.84	0.75	2.18	3.42
Synergi Fusion RP (Phenomenex)	With t_0	0.04	With t_0	0.16	0.04
TSKGel Amide-80 (Tosoh)	1.27	3.12	3.45	10.20	12.80
PolysulfoethylA (PolyLC)	0.77	2.08	3.14	15.02	16.69
ZIC-HILIC (SeQuant)	0.89	2.09	3.24	9.54	12.63
Chromolith performance Si (Merck)	0.29	0.76	0.61	2.26	2.04

[a)] Conditions see Section 2.

Table 5. Chromatographic results for the HILIC test for vitamins (for structures and log *P*/log $D_{8.0}$ see Fig. 9)[a)]

	$k_{thiamine}$	$k_{pyridoxine}$	$k_{riboflavine}$	$k_{ascorbic\ acid}$	$k_{nicotinic\ acid}$
In-house developed RP/WAX columns					
RP/WAX-AQ360 (high capacity) (Fig. 1a)	0.09	1.17	2.17	n/d	34.37
RP/WAX-DMAE (Fig. 1c)	0.17	0.88	1.03	15.03	16.32
RP/WAX-BAMQO (Fig. 1d)	0.73	0.57	0.40	7.46	15.04
Commercial/comparison columns					
Acclaim Mixed Mode WAX-1 (Dionex)	0.17	1.11	1.66	n/d	31.14
Uptisphere 5 MM3 (Interchim)	0.60	0.13	0.13	5.27	3.03
Primesep B2 (Sielc)	3.92	0.36	0.04	0.04	0.73
Obelisc R (Sielc)	10.11	0.78	0.78	2.73	10.11
Obelisc N (Sielc)	n/d	6.65	1.15	1.15	0.66
Chiralpak QN-AX (Chiral Technologies)	1.17	0.41	0.32	n/d	3.52
Luna NH_2 (Phenomenex)	0.95	2.64	3.10	28.74	24.90
BioBasic AX (Thermo Scientific)	0.43	0.43	0.89	n/d	10.88
Synergi Fusion RP (Phenomenex)	14.94	0.11	With t_0	With t_0	0.44
TSKGel Amide-80 (Tosoh)	43.54	1.49	4.77	20.70	5.49
PolysulfoethylA (PolyLC)	26.81	2.73	3.10	n/d	2.73
ZIC-HILIC (SeQuant)	29.30	1.20	2.20	35.13	4.89
Chromolith performance Si (Merck)	28.20	0.65	0.65	1.41	1.85

[a)] Conditions see Section 2. n/d, not detected.

low to accomplish reasonable retention for the xanthins and somewhat surprisingly the same seems to be the case for Obelisc N which is supposed to be a polar material with normal-phase characteristics suitable for HILIC separations of polar compounds. The retention characteristics for Obelisc R is somewhere in-between that of the RP/WAX-AQ360 and Primesep B2 columns.

A comparison of the chromatograms acquired for the nucleosides test mixture is depicted in Fig. 11. None of the columns obeyed the elution order that was expected from log *D* values (adenosine **1** < thymidine **2** < uridine **3** ~ guanosine **4** < cytidine **5**) (note, numbering corresponds to the elution order as expected from log *D* values). It is striking that the compounds with higher hydrogen-donor/acceptor numbers are stronger retained than expected from the log *D* values leading to altered elution orders following thymidine **2** < adenosine **1** < uridine **3** < cytidine **5** < guanosine **4** on most of the tested columns (adenosine shifted to elute after thymidine and guanosine shifted to elute after cytidine). On Luna NH_2, Biobasic AX, and Obelisc R column, adenosine and uridine are not sufficiently resolved and coelute, respectively.

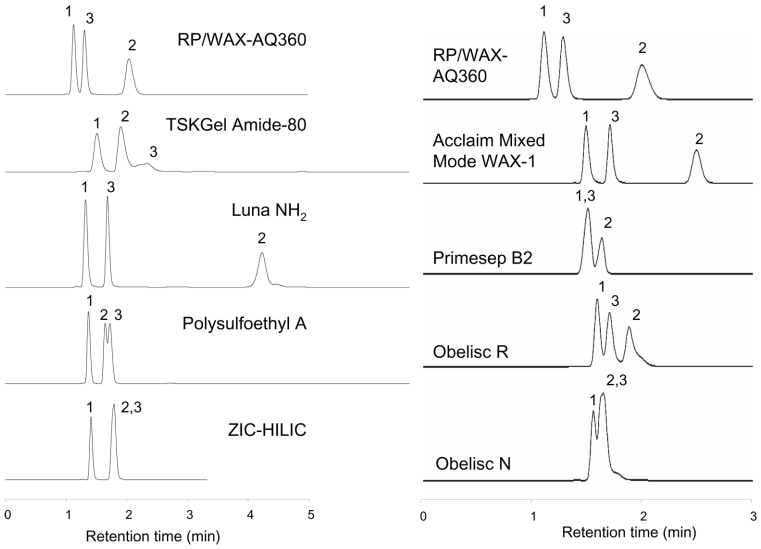

Figure 10. Chromatograms of the HILIC test for xanthins. Experimental conditions see Section 2. Solutes: **1**, caffeine; **2**, theophylline; **3**, theobromine. (Note, numbering corresponds to the elution order as expected from log *D* values).

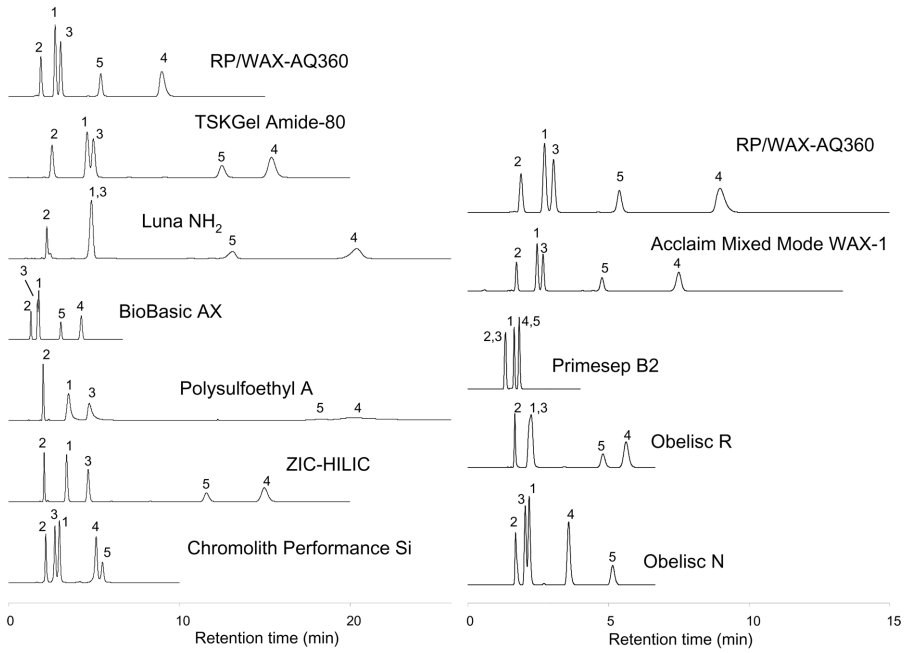

Figure 11. Chromatograms of the HILIC test for nucleosides. Experimental conditions see Section 2. Solutes: **1**, adenosine; **2**, thymidine; **3**, uridine; **4**, guanosine; **5**, cytidine. (Note, numbering corresponds to the elution order as expected from log *D* values).

Most remarkable are, however, the fundamentally altered elution profiles on the silica monolith Chromolith Performance Si and the Obelisc N column. Elution orders are reversed for the adjacent adenosine/uridine as well as the cytidine/guanosine peak pairs. Moreover, the latter peak pair is unresolved on Primesep B2 and the uridine peak coelutes with the thymidine peak. All these shifts in elution order and elution profiles, respectively, can hardly be explained on the basis of a partition mechanism and rather specific interactions between solutes and functional groups of the chromatographic ligands of the stationary phase have to be invoked to explain such specific elution profiles (*vide infra*).

Even more pronounced peculiarities in the elution profiles of the distinct stationary phases are found for the vitamins test mix (Table 5). To some extent, this is not surprising because this test sample contains ionic constituents which are expected to show strongly deviating retention profiles on stationary phases with cationic, anionic, zwitterionic, and neutral chromatographic bonding chemistries. It is beyond the scope to further discuss all these separation results in detail at this place. A few characteristic points will be outlined later. In general, the retention profiles are, however, once more a strong indication for the importance of specific interaction forces between complementary functional groups of

the solutes and stationary phase ligands while mere partition mechanisms fail to explain altered retention profiles and shifts in elution orders on the distinct stationary phases.

3.2.2 Mechanistic insights and assessment of complementarity

It is a common perception that the retention mechanism in HILIC is due to partition of solutes between an adsorbed water layer (representing a polar stationary phase) and the less polar usually ACN-rich mobile phase [3]. Anomalities in view of this partitioning model have been explained by superimposed adsorption mechanisms originating from specific interactions between functional groups of the solutes and the stationary phase. Such a partitioning model was earlier also under debate for RP-HPLC [34], however, has now been dropped in favor of a more differentiating multiple interaction model [1].

If partitioning was the driving force in the present HILIC separations (i) it would be expected that log k values roughly linearly drop with an increase in log D which is a solute descriptor characterizing its aqueous-organic two-phase distribution property [35], and (ii) plots of retention factors on HILIC column A *versus* retention factors on HILIC column B ($\log k_A$ *vs.* $\log k_B$) should yield straight lines with only slight scatter in the order of experimental errors because stationary phase (adsorbed water layer) as well as mobile phase are supposed to have identical compositions in the two distinct phase systems (ion distribution profiles in contrast may be different for stationary phases with distinct surface charge characteristics). In case two tested columns would exhibit identical phase ratios, a slope of unity should result. Since it is unlikely that two different stationary phases tested herein give identical phase ratios, the trend line is supposed to deviate from unity, reflecting merely different phase ratios of the two columns while still a nearly perfect correlation should be obtained if a partitioning mechanism prevails. Hereafter, the presented results are analyzed in view of these considerations.

Simple regression analysis with log D values (calculated by ACD for pH 8) as independent variable and log k values of each column as dependent variable has been performed on the HILIC data obtained with the mobile phase composed of 90% ACN and 10% ammonium acetate buffer. Table 6 summarizes the percent of variability in the dependent data (as measured by the r^2 statistics) explained by the model using log D as a single parameter in the regression equation. As can be seen from Table 6 about 30 – 70% or after removal of outliers about 40 – 90% of the variability in the log k values can be explained by the model relating retention with the partition coefficient log D. Thus, there seems to be indeed a component in the retention mechanism which points towards partitioning. Yet, such statistics on the other hand also implies that there is another component contributing to retention as well. In fact, Jinno and coworkers proposed QSPR models for adrenoreceptor agonists obtained by MLR with several predictor variables including log D but also others such as the hydrogen-donor number, desolvation energy for octanol, and the total absolute atomic charge [36]. In view of this argumentation, a partition mechanism is rather disapproved than unequivocally approved especially if it is considered that log D is of course also a general solute descriptor for the hydrophilicity of molecules and does not distinguish whether the retention is due to interactions with an adsorbed polar aqueous phase or polar functional groups on the stationary phase.

Table 6. Percentage of variability in retention factors (as measured by r^2) explained by log D

Stationary phase	% of Variability (r^2) explained by log D (log k *vs.* log D)	
	All solutes	Without outliers
RP/WAX-AQ360	49	73
RP/WAX-DMAE	35	72
RP/WAX-BAMQO	34	54
Acclaim MM WAX 1	53	74
Uptisphere MM3	29	66
PrimesepB2	8	59
Obelisc R	30	42
Obelisc N	7	38
Chiralpak QN-AX	60	80
Luna NH2	42	57
BioBasic AX	69	89
Synergi Fusion RP	5	0
TSKGel Amide-80	38	82
PolysulfoethylA	50	75
ZIC-HILIC	42	72
Chromolith performance Si	12	35

In Fig. 12 the retention factors of ZIC-HILIC, Luna NH_2, RP/WAX-AQ360 and Acclaim Mixed-Mode WAX-1 are plotted against the retention factors of TSKGel Amide-80. The TSKGel Amide-80 column is used here as a reference column because it has a neutral surface bonding and as long as silanol interactions are unimportant a possibly existing partition mechanism should not be blurred by superimposed ionic interactions.

Figure 12a displays the correlation between retention factors of ZIC-HILIC and TSKGel Amide columns. As can be seen there is a strong correlation between the retention data on these two columns despite the completely different functional groups incorporated in their chromatographic ligand. The TSKGel Amide phase features H-donor/acceptor groups while the ZIC-HILIC phase has incorporated a quaternary ammonium group (without any H-donor) and a sulfonate group with H-acceptor

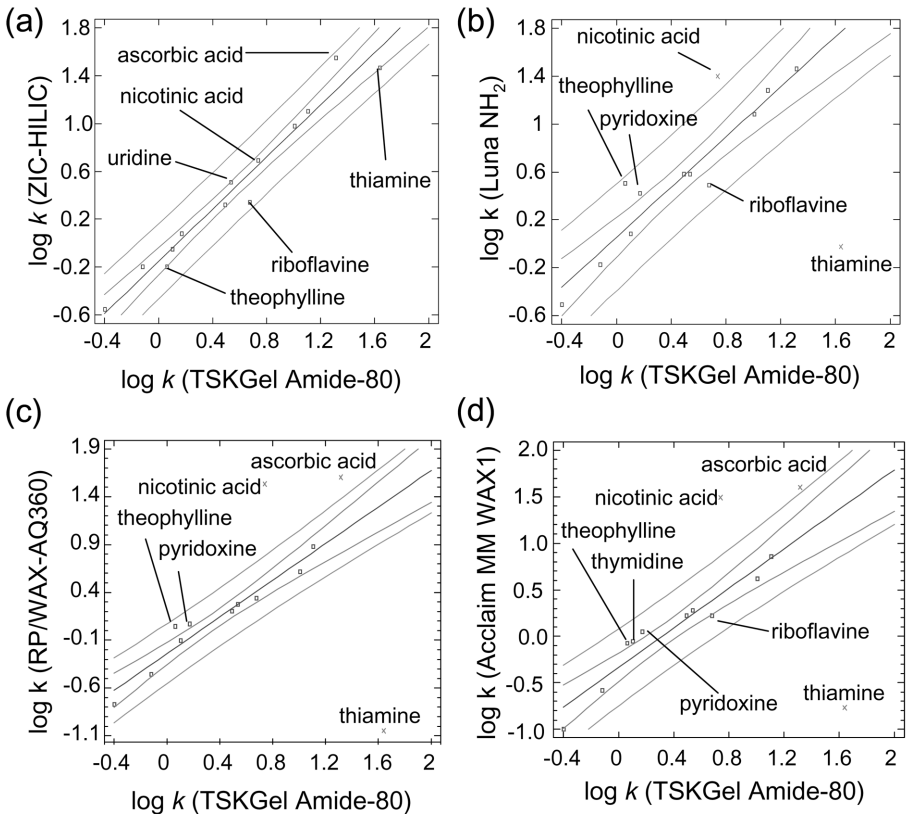

Figure 12. Orthogonality plots of log *k* values from HILIC tests of TSKGel Amide-80 *versus* (a) ZIC-HILIC, (b) Luna NH_2, (c) RP/WAX-AQ360, and (d) Acclaim Mixed-Mode WAX-1. Besides the fitted model the plots show the confidence (inner lines) and prediction limits (outer lines).

properties. Solely ascorbic acid which is stronger retained on the ZIC-HILIC column and thiamine as well as riboflavin which are stronger retained on the TSKGel Amide column deviate significantly from the trend line. It seems that ionic interactions (probably with residual silanols in case of TSKGel Amide) are responsible for these deviations.

Similar correlations of retention factors of Luna NH_2, RP/WAX, and Acclaim Mixed-Mode WAX-1 with those of TSKGel Amide can be found (Figs. 12b – d). Slightly stronger scatter is obtained for all of these phases. Most significant deviations from the trend line are, not surprisingly, observed especially for ionic constituents in the sample being indicative for superimposed electrostatic interactions. Thiamine is eluted much earlier on the amino- and mixed-mode phases than may be expected from a mere partition mechanism due to electrostatic repulsion. Conversely, acids like ascorbic and nicotinic acid are stronger retained due to superimposed anion-exchange retention processes. The fact that correlations exist for very distinct stationary phase chemistries would be in favor for a partition model of retention. However, outliers scatter more strongly for some of the tested solutes (*e.g.*, riboflavin, pyridoxine, theophylline) and the above-discussed reversals of elution order are indications that specific adsorption mechanisms such as arising from solute – sorbent interactions are undoubtedly of importance [37].

Overall the data are not absolutely conclusive. However, while specific retention patterns and reversals of elution orders on distinct columns may readily be explained on the basis of an adsorption mechanism, this is not possible for a partitioning model. The difficulty may arise from the fact that polar functional groups of the sorbents are supposed to be strongly solvated under aqueous-organic conditions and specific interaction of solutes with these moieties requires their desolvation which is energetically unfavorable. The energy cost for desolvation has to be compensated for, by an energy gain upon interaction. If the energy balance is unfavorable the interaction does not probably occur, yet the solute may interact with the solvent shell, *e.g.*, through a network of hydrogen bonds which is also often seen in hydrated crystal structures of ligand protein interactions and it might be a matter of a more semantic debate, whether such a scenario is to be termed adsorption or partitioning. Entropic contributions from reordering of the system have to be considered as well. In view of this, other models based on thermodynamics or extra-thermodynamics such as linear free energy relationship (LFER) models [38] or extended linear solvation energy relationship (LSER) models [1, 39] may probably better explain the mechanism in HILIC, as they do better account for all the retention contributions. A safe conclusion, though, is difficult. Moreover, to what extent the present data set

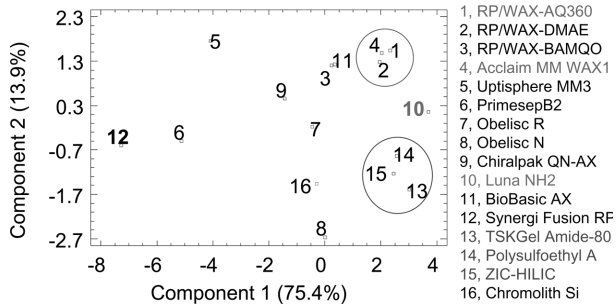

Figure 13. Results of the PCA on the HILIC-elution data: Score plot of the two first principal components.

is representative is also to be questionized and thus an extended set of solutes is currently tested on these columns to allow for more safe conclusions.

3.2.3 Principal component analysis on HILIC data

Like above, the HILIC data matrix was subjected to PCA to figure out similarities and dissimilarities of the tested columns with regard to their retention and separation characteristics. Figure 13 shows the scatter plot of the first two components which explain together about 89% of the variability in the dependent variable. By detailed inspection of the results it may be argued that component 1 lumps together information on the hydrophilicity/lipophilicity of the stationary phases and component 2 on their charge character.

As can be seen from the score plot in Fig. 13, the in-house developed RP/WAX and the Acclaim Mixed-Mode WAX-1 phases are grouped together as they behaved similar in the HILIC screening. In contrast, TSKGel Amide-80, ZIC-HILIC, and Polysulfoethyl A gave quite dissimilar retention profiles in comparison to the RP/WAX phases in the HILIC tests of the presently investigated solutes as may be derived from their far distant clustering on the latent variable of component 2. Luna NH_2 appears to show also strong HILIC character, yet it is dissimilar to both RP/WAX phases and the other above-mentioned HILIC phases. It is also striking that Obelisc N and Chromolith Performance Si are closer to the ZIC-HILIC/TSKGel Amide cluster while Biobasic AX, Obelisc R, and Chiralpak QN-AX are closer to the RP/WAX cluster. Primesep B2 and Uptisphere MM3 on the other hand are more similar to the Synergi Fusion RP column. Overall, these trends make sense, yet care has to be taken if it is attempted to transmit the results directly to other solute sets as the present data matrix is somehow restricted. The data set is presently extended in our laboratory focusing in particular to endogenous metabolites to confirm the present findings.

4 Concluding remarks

A set of in-house developed RP/WAX stationary phases combining long chain alkyl groups with weak anion exchange moieties represented by tertiary amines as well as polar embedded amide and sulfide groups were chromatographically evaluated in comparison to various commercially available stationary phases under RP conditions (*i.e.*, employing aqueous-rich hydroorganic eluents) as well as HILIC elution modes (*i.e.*, ACN-rich hydroorganic eluents). The commercial columns tested included a polar RP material (Synergi Fusion RP), two amino phases (Luna NH_2, BioBasic AX), various mixed-mode phases (Acclaim Mixed-Mode WAX-1, Uptisphere MM3, Primesep B2, Obelisc R, Obelisc N), a chiral anion-exchange phase with mixed-mode character (Chiralpak QN-AX), two typical HILIC phases (TSKGel Amide-80, ZIC-HILIC), a mixed-mode HILIC/cation exchanger (Polysulfoethyl A), and a silica monolith (Chromolith Performance Si). The major findings from these chromatographic evaluations can be summarized as follows:

(i) When employed with RP-conditions (40% ACN) the in-house developed RP/WAX phases as well as commercial RP/WAX phases like Acclaim Mixed-Mode WAX-1, Primesep B2, and Obelisc R exhibited slightly lower hydrophobic selectivity as measured by a methylene selectivity α_{CH_2} of ~1.5 compared to a polar embedded RP phase (such as Synergi Fusion RP) with α_{CH_2} of ~1.8. The Uptisphere MM3 stationary phase resembled the Synergi Fusion RP with respect to methylene selectivity and the low molar nitrogen coverage points towards a polar end-capped phase with cationic groups.

(ii) The amino phases lacked hydrophobic selectivity and showed, in contrast to the RP/WAX phases, stronger retention for the hydrophilic than the more hydrophobic acidic test compound.

(iii) PCA confirmed that the tested in-house RP/WAX columns as well as the Acclaim Mixed-Mode WAX-1 phase resemble each other in their chromatographic characteristics, while a decrease in mixed-mode character due to lower ion-exchange capacity could be identified from Obelisc R > Primesep B2 > Uptisphere MM3.

(iv) The in-house developed RP/WAX phases, and the commercial Acclaim Mixed-Mode WAX-1 likewise, also showed applicability for HILIC while such a multimodal applicability was not found for TSKGel Amide-80, ZIC-HILIC, Amino phases, or Synergi Fusion RP.

(v) PCA on the HILIC data revealed that the RP/WAX phases behave dissimilar to the TSKGel Amide-80, ZIC-HILIC, and Polysulfoethyl A and show distinctive retention and selectivity profiles in HILIC mode. Hence they may be regarded as complementary to these commercial stationary phases.

(vi) On the basis of the afforded retention data mechanistic considerations for the HILIC elution mode were

undertaken by correlations of retention patterns with solute descriptors as well as orthogonality plots of distinct columns. Although at a first course glance, decent support for a partition model of solute retention in HILIC could be invoked, a closer inspection suggested to depart from such a model. Rather the retention profiles and in particular their fine patterns such as reversed elution orders on stationary phases with distinct groups point toward involved specific interactions which are in line with an adsorption mechanism.

From the present study it becomes evident that the mixed-mode RP/WAX phases represent a valuable complement to the stationary phases on the market with polar selectivity and applicability profiles.

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The authors declared no conflict of interest.

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ADDENDUM # 4

Manuscript (MS4)

Under review

Ganoderma species discrimination by liquid chromatographic fingerprint analysis: A study on column classification and stationary phase effects in hydrophilic interaction chromatography and reduction of sample misclassification rate by additional use of reversed-phase chromatography

Yi Chen[1,2§], Wolfgang Bicker[2]*, JunYan Wu[2], Ming Yong Xie[1]**, Wolfgang Lindner[2]

[1]State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, People´s Republic of China

[2]Christian Doppler Laboratory for Molecular Recognition Materials, Department of Analytical Chemistry and Food Chemistry, University of Vienna, Waehringer Strasse 38, A-1090 Vienna, Austria

* Corresponding author:
c/o FTC-Forensic-Toxicological Laboratory Ltd., Simmeringer Hauptstrasse 24, A-1110 Vienna, Austria
Tel.: +43-1-74040-2831, Fax: +43-1-74040-2839
E-mail address: wolfgang.bicker@univie.ac.at (W. Bicker)

** Additional corresponding author:
Tel.: +86-791-3969009, Fax: +86-791-3969069
E-mail address: myxie@ncu.edu.cn (M. Y. Xie)

[§]...Experimental work was performed during a research stay of author Y. C. at the Department of Analytical Chemistry and Food Chemistry, University of Vienna.

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Abstract

Acetonitrile-water extracts of several *Ganoderma* species – a mushroom being used in Traditional Chinese Medicine – were analysed by liquid chromatography – UV detection in hydrophilic interaction chromatography (HILIC) and reversed-phase (RP) elution modes. A set of six polar stationary phases was used for HILIC runs. These columns had remarkably different separation properties under binary gradient conditions as evinced by hierarchical cluster analysis on retention patterns of seven test compounds. Complementary measurements of RP chromatograms were carried out on a C_{18} packing. Injection precision (n = 5) and intra-day precision (n = 5) were each < 2.0 % RSD (HILIC) and < 0.7 % RSD (RP) for relative retention times of main characteristic peaks of a sample extract while for relative peak areas RSD values were max. 6.8 %. Repetitive analysis (n = 7) of a processed sample stored in the autosampler tray for 48 h was used to confirm within-sequence sample stability. Eleven *G. lucidum* samples served as training set for the construction of column-specific simulated mean chromatograms. Validation with twelve samples comprising *G. lucidum, G. sinense, G. atrum*, and *G. tsugae* by correlation coefficient based similarity evaluation of peak patterns showed that a discrimination of *G. lucidum* from other *Ganoderma* species by means of chromatographic fingerprints is conceptually possible on all columns, except of a bare silica packing. The importance of the combined use of RP and HILIC fingerprints to improve the rate of correct sample classification was demonstrated by the fact that each one *G. sinense* specimen was wrongly assigned being *G. lucidum* by all HILIC fingerprints but not the RP fingerprint and *vice versa*. The present data revealed that (i) the analysis of complex biological materials by quasi orthogonal chromatographic modes such as HILIC and RP may deliver more discriminative information than single-mode approaches which strengthens the reliability of fingerprint-based sample classification and (ii) different retention and selectivity characteristics of polar bonded silica packings in the HILIC elution mode may only have a minor impact on chemometric sample discrimination capabilities in such kind of pattern-oriented metabolomics separation problems.

1 Introduction

Positive effects on human health exerted by the intake of certain plant and mushroom preparations are known since ancient times and this knowledge forms the basis for many health-promoting and therapeutic concepts in the framework of Traditional Chinese Medicine (TCM). Within the past decades TCM gained popularity throughout the world and this was accompanied by an increased commercialisation of plant/fungal preparations [1]. Identification, standardisation, and quality control of biological materials and extracts prepared thereof are major tasks faced by manufacturers and health authorities as well [2,3]. Quite often it is impossible to assign the observed pharmacological (but also toxicologically relevant) properties of TCM preparations to the presence of certain active compounds and mixture-effects seem to be common. For these reasons, one particular analytical challenge in the adequate characterisation of traditional remedies resides in the need for a quasi-holistic approach.

To circumvent comprehensive compound identification and absolute quantification in routine work, which would for instance require information-rich detection methods such as multistage mass spectrometry or the availability of reference standards, while still coping with the complexity of biological materials, a “fingerprint analysis” is of viable means [3-13]. Thereby, neat or processed samples are characterised either directly by spectroscopic methods (e.g. infrared spectroscopy, nuclear magnetic resonance spectroscopy) or by separation techniques (chromatography, capillary electrophoresis) hyphenated with selective (e.g. UV/VIS, mass spectrometry) or almost non-selective (e.g. evaporative light scattering) detectors. Chemometric tools are then being applied on raw data in order to discriminate samples by comparison with a reference fingerprint (pattern approach). Fingerprint analysis experienced considerable popularity within the past years and, meanwhile, it is internationally largely accepted for (batch-)quality control of herbal and fungal preparations. In addition to that, it can also used for the differentiation of samples by species or geographical origin.

Separation techniques reduce sample complexity by spreading the information content in the time domain and therefore they are particularly valuable for fingerprint analysis. Yet, very polar but also non-polar as well as charged and non-charged compounds are usually present in biological specimens and, intrinsically, the analyte profile actually coverable by a single separation method is limited. For example, the use of reversed-phase (RP) columns in liquid chromatography (LC) is suitable for the separation of moderately polar to non-polar compounds, but very hydrophilic solutes will (co-)elute in the early part of the chromatogram. Consequently, fingerprint information may likely get lost for such compounds and the RP peak profile basically reflects the sample composition with respect to hydrophobic solutes. The

selectivity window of RP chromatography may be increased by using stationary phases which have an ionisable or permanently charged group incorporated in the hydrophobic ligand [14-17]. This allows for the addition of strong electrostatic molecular distinction to the base RP separation process. Compared to single-mode RP packings such kind of ´mixed-mode´ columns have their application range extended to polar charged compounds. However, under typical RP elution conditions low retention is still obtained for non-charged polar solutes.

Complementary, one may use these mixed-mode materials [14,18,19] or any type of polar packings [20-22] in the hydrophilic interaction (HILIC) elution mode. HILIC is targeted towards the retention of polar compounds in general. In contrast to RP eluents, HILIC mobile phases are characterised by being rich in (non-protic) organic solvent (usually acetonitrile), they have water (or other polar protic solvents [23]) added as the strong eluting modifier, and they usually contain mM amounts of organic salts (e.g. ammonium salts of formic or acetic acid). The mechanisms that generate retention on polar chromatographic materials when being operated under such elution conditions are not yet fully elucidated. A plausible model is that analytes partition between a dynamically adsorbed polar protic layer and the less-polar mobile phase and that, additionally, adsorptive interactions (e.g. hydrogen bonding) develop between analytes and ligand functionalities or the sorbent surface.

Thus, the compound spectrum available for the characterisation of complex biological samples may be considerably extended by using a dual chromatographic approach in which (quasi-)orthogonal separation modes such as RP and HILIC are combined (on-line or off-line) [22,24-30]. Surprisingly, chromatographic fingerprinting of plant/fungal samples by LC methods is almost exclusively based on the use of RP columns, and only very recently the characterisation of an herbal extract by both RP and HILIC was reported [31]. In the course of the present study it was regarded necessary to explore besides RP in some more detail the potential of HILIC for being applicable for such kind of analytical problem.

In contrast to the stationary phases typically used for RP separations, there is much greater diversity in the phase chemistry of chromatographic materials applicable in the HILIC elution mode. Polar column packings range from non-functionalised silica to silicas bonded with different polar non-chargeable, or chargeable, or permanently charged ligands (anionic, cationic, zwitterionic). This diversity is reflected in widely varying retention and selectivity characteristics, especially when charged solutes and charged chromatographic ligands are considered [14]. While such inter-column differences provide flexibility in target-orientated method development their impact on pattern recognition-based non-targeted analytical problems such as chromatographic fingerprinting is difficult to estimate. One particular aim of the present work was therefore to investigate HILIC stationary phase effects in the framework

of fingerprint-based sample discrimination capability and to compare the results to a chemometric column classification obtained with a small set of compounds. For this purpose six polar columns were comparatively tested and the analysis of *Ganoderma* extracts served as a real-world example.

Ganoderma is a mushroom consisting of > 200 species to some of which health-promoting and therapeutic properties are attributed [32-36]. In particular, preparations made of strains of *G. lucidum* and related species have a long history as dietary supplements and curing agents in Asian folk medicine. Positive action on disease states such as migraine, diabetes, asthma, hypertension, and hepatitis are reported. Recently, researchers became strongly interested in the pro-apoptotic and anti-proliferative effects exerted by *Ganoderma* preparations which may hold promise in cancer therapy.

Fingerprint analysis of *Ganoderma* for the purpose of quality control or the discrimination of samples by geographical origin, species or strain was in the scope of a few earlier studies [37-41]. In case LC was employed it was limited to the use of RP packings [38,39,41]. Main compound classes identified in mycelia and fruiting bodies of *Ganoderma* include non/less polar (e.g. triterpenoids) but also very polar substances (e.g. polysaccharides, nucleosides). Thus, a combined sample characterisation by HILIC and RP seems to be particularly advantageous in order to cope better with this high degree of sample complexity. In the present work, acetonitrile-water extracts of *Ganoderma* specimens were analysed by LC-UV separately in HILIC and RP elution modes to evaluate orthogonality of the obtained fingerprints from a practical point of view. The peak profiles were input data for the development of a chemometric approach to probe the differentiation of *G. lucidum* from some other *Ganoderma* species by similarity analysis of chromatograms.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

HPLC grade acetonitrile (ACN) was purchased from VWR International (Vienna, Austria). Ammonium acetate (analytical grade) and chromatographic test compounds for HILIC column characterisation (benzyltrimethylammonium chloride, cytidine, guanosine, 4-hydroxybenzoic acid, 4-hydroxybenzenesulfonic acid, tyramine, tyrosine) were supplied by Sigma-Aldrich (Vienna, Austria). Water was bidistilled in-house.

2.1.2 Chromatographic set-up

All chromatographic runs were carried out on a 1200 Rapid Resolution LC system from Agilent Technologies (Waldbronn, Germany) equipped with a diode array detector. Instrument control and data acquisition were carried out with the Chemstation software (version B.04.01).

Commercially available 150 x 4.6 mm ID columns packed with Luna HILIC (Phenomenex, Aschaffenburg, Germany), Polysulfoethyl A (PolyLC, Columbia, MD, USA), TSKGel Amide-80 (Tosoh Bioscience, Stuttgart, Germany), and ZIC-HILIC (SeQuant, Umea, Sweden), as well as 150 x 4.0 mm ID stainless steel columns packed in-house with commercial bare silica (Daisogel supplied by Daiso Chemical, Osaka, Japan) and a home-made polar functionalised Daisogel material named TGO [23,42] were used for runs in the HILIC elution mode. Some characteristic properties of these polar phases are summarised in Table 1.

<Table 1>

The columns were protected by a 10 x 2.1 mm ID pre-column from Thermo Fisher Scientific (Waltham, MA) containing a C_4-RP material. Chromatographic runs in the RP elution mode were conducted on a Zorbax SB-C_{18} column (100 x 2.1mm ID, 1.8 µm particle size, 80 Å pore size) from Agilent Technologies which was protected by an in-line filter (2.0 mm ID, 0.2 µm pore size) from the same manufacturer.

2.1.3 Ganoderma samples

The fruiting bodies of twenty-three *Ganoderma* batches comprising *G. lucidum*, *G.atrum*, *G. sinense*, and *G. tsugae* were collected from different locations of China in the period of May 2007 – May 2008 (Table 2).

<Table 2>

Morphological species identification was carried out by Dr. Zhi-hong Fu (Jiangxi University of Traditional Chinese Medicine, China). Samples for chromatographic analysis were collected from the pileus of cultivated fruiting bodies, except of specimens no. 13, 14, 19, 21, and 22 which were taken directly from *Ganoderma* fruiting bodies grown in nature. Sample no. 15 consisted of broken spores. The collected specimens were dried at 60 °C for 12 h and cut into small pieces.

2.2 Methods

2.2.1 Characterisation of polar column packings in the HILIC elution mode

Separation properties of various polar columns (Table 1) in the HILIC elution mode were

evaluated using a set of seven test solutes (for structural formulas see Figure 1).

<Figure 1>

Elution was carried out using a binary gradient with 10 mM ammonium acetate in ACN / water (50:50; *v*/*v*) (solvent channel A) and ACN / water (95:5; *v*/*v*) (solvent channel B). The time program was as follows: 0 min: 100 % B, 5 min: 100 % B, 40 min: 0 % B, 45 min: 0 % B, 45.1 min: 100 % B, 60 min: 100 % B. In order to standardise the gradient conditions for the individual columns (column hold-up volume ranging from 1.41-1.81 mL as determined with the elution time of toluene using 100 % B) flow rates between 1.00 and 1.29 mL min^{-1} were adjusted to end up with an equivalent flow velocity of 1.8 mm s^{-1} for each column. To speed up column re-equilibration the flow rate was each increased by a factor of two between 45 and 55 min. The column compartment temperature was kept at 30 °C and the injection volume was 2 µL. Chromatograms were recorded at 254 nm (8 nm bandwidth) using 450 nm (100 nm bandwidth) as reference wavelength.

Hierarchical cluster analysis (HCA) on retention times 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.

2.2.2 Ganoderma sample preparation

1.0 g of sample was transferred to a 30 mL volumetric flask. 20 mL of ACN / water (50:50; *v*/*v*) were added and the mixture was extracted twice at 50 °C for each 60 min. Subsequently, suspensions were filtered through filter paper and the whole filtrate was evaporated to dryness at 50 °C and 50 mbar. The dried extract was re-dissolved in 1.0 mL ACN / water (50:50; *v*/*v*) and the resulting solution was passed through a 0.20 µm nylone membrane filter (Sigma-Aldrich, Vienna, Austria) prior transfer to a glass autosampler vial.

2.2.3 Chromatographic analysis of Ganoderma extracts

Ganoderma sample extracts were analysed in HILIC and RP elution modes. HILIC elution conditions were fully according to those detailed in section 2.2.1 and each sample extract was run on each polar column. RP runs were conducted on the C_{18} column using a binary gradient with 10 mM ammonium acetate in ACN / water (1:99; *v*/*v*) (solvent channel A) and 10 mM ammonium acetate in ACN / water (95:5; *v*/*v*) (solvent channel B) as mobile phases. The time program was as follows: 0 min: 10 % B, 1.5 min: 10 % B, 2 min: 30 % B, 15 min: 33 % B, 20 min: 35 % B, 25 min: 100 % B, 45 min: 100 % B, 45.1 min: 10 % B, 60 min: 10 % B. The flow rate was kept at 0.20 mL min^{-1} for the first 15 min, then increased to 0.30 mL min^{-1} within 5

min and kept at 0.30 mL min^{-1} for 25 min before re-equilibrating for 15 min at 0.20 mL min^{-1}. The column compartment temperature was kept at 50 °C and the injection volume was 2 µL. Chromatograms were recorded at 254 nm (8 nm bandwidth) using 450 nm (100 nm bandwidth) as reference wavelength.

2.2.4 Method validation

Ganoderma sample no. 14 (cf. Table 2) was used to validate the HILIC and RP methods with respect to the relative standard deviation (%RSD) of relative retention times (RRT) and relative peak areas (RPA) of some characteristic peaks. In the HILIC elution mode, the ZIC-HILIC column was used a representative for the other polar columns packings. Injection precision was determined by replicate injection (n = 5) of a processed sample. Injection of five different sample solutions prepared independently was used to assess intra-day method precision. Stability of processed sample solutions during storage in the autosampler was checked by repetitive injection over a period of 48 h (0, 2, 4, 6, 8, 24, 48 h post extraction).

2.2.5 Chemometric species differentiation

Similarity analysis of chromatograms was carried out using the software "Similarity Evaluation System for Chromatographic Fingerprints of Traditional Chinese Medicine" (Version 2004 A), which is recommended by the National Commission of the Chinese Pharmacopoeia. The raw chromatographic data were imported in the form of *.cdf-files into the software. In order to focus on the most discriminative information time windows of 0-32 min (HILIC) and 5-42 min (RP), respectively, were used for chemometric analysis. Variations in retention times were corrected by the target peak automatic alignment procedure of the software. A mean chromatogram of *G. lucidum* (chromatographic fingerprint consisting of the "common" peak pattern) was constructed from samples no. 1-11 (training set; see Table 2). Samples no. 12-23 comprised different *Ganoderma* species and served as validation set for chromatographic fingerprint-based species discrimination. Correlation coefficients of individual sample chromatograms relative to the simulated mean chromatogram were calculated according to eq. (1).

$$r = \frac{\sum_{i=1}^{n}(x_i - x_m)(y_i - y_m)}{\sqrt{\sum_{i=1}^{n}(x_i - x_m)^2 \sum_{i=1}^{n}(y_i - y_m)^2}} \quad (1)$$

where x_i and y_i represent the peak areas of the ith peak in the individual chromatogram x and the calculated reference chromatogram y, and n is the total number of peaks in the chromatograms. x_m and y_m are median values of peak areas for n peaks in chromatograms x and y, respectively.

Correlation coefficients were used as descriptor for similarity of the chemical character of the various *Ganoderma* samples.

3 Results and Discussion

3.1 Characterisation of polar column packings in the HILIC elution mode

The dominant interactions in the HILIC elution mode (partitioning/adsorption, weak/strong electrostatic interactions) may strongly depend on the type of analyte, the type of stationary phase, and the chosen elution conditions. This complexity sometimes hampers straightforward method development. The design of a HILIC column classification test able to adequately characterise the whole spectrum of interactive mechanisms becomes a particularly difficult task as well. Not least due to this reason and despite the still growing interest in HILIC, systematic column classifications did not yet appear in the literature.

In the framework of the present study it was of interest to get an idea about the separation properties of the selected polar columns towards a defined set of compounds in order to have complementary information to the non-targeted fingerprint data. Retention times of weak/strong bases (tyramine, benzyltrimethylammonium chloride), weak/strong acids (4-hydroxybenzoic acid, 4-hydroxybenzenesulfonic acid), a zwitterion (tyrosine), and two essentially non-charged solutes (cytidine, guanosine) were measured. Structural formulas of the test compounds are depicted in Figure 1. Elution conditions of this simple column classification approach were identical to those used for *Ganoderma* sample extract analysis (10 mM ammonium acetate in acetonitrile-water gradient, no pH adjustment).

The investigated chromatographic materials involved bare silica and five silicas bonded with different polar functionalities (Table 1). One packing, named TGO, was prepared in-house. This stationary phase is characterised as silica functionalised with a C_5 ligand which carries a diol motif and a sulfoxide functionality [23,42]. Two of the commercial bonded packings are described by the manufacturers to be of ethylene-bridged diol-type (Luna HILIC) and carbamoyl-type (TSKGel Amide-80), respectively. Like the TGO bonding, also the two latter modifications are considered to be non-charged. One column contains a zwitterionic sulfobetaine-type ligand bonded to silica (ZIC-HILIC). As a representative for the considerable number of commercially available chargeable HILIC materials a poly(2-sulfoethyl aspartamide)-type bonded phase (Polysulfoethyl A), i.e. a hydrophilic strong cation exchanger, was selected.

The highly polar zwitterion tyrosine (log D = -2.4 – -2.1 calculated at 25 °C in a range of pH 5-

7; data taken from SciFinder scholar database whereby all values were calculated with ACD/Labs Software V8.14, Advanced Chemistry Development Inc., Toronto, Canada) was eluted latest on all columns (retention time t_R window 16-21 min). Non-charged cytidine (log $D^{pH\ 5\text{-}7}$ = -1.9) typically eluted 0.3-2 min before guanosine (log $D^{pH\ 5\text{-}7}$ = -1.7; t_R window 13-20 min) except of the bare silica material where reversal of the elution order was observed (cf. our earlier results on this analyte pair [42]). If the interpretation of the retention patterns on the set of polar columns is limited to these three analytes it appears that compound polarity in terms of the calculated log D value is a suitable descriptor for the extent of retention which may thus indicate a partitioning-based mechanism. However, a pronounced scatter in the retention pattern of anionic and cationic solutes, which overall delivered a specific elution order for each column, showed that such a concept is far too simple to adequately describe the HILIC retention process.

To visualise similarities in the separation patterns of the various columns chemometric data treatment by HCA was performed on retention times (Figure 2).

<Figure 2>

Several clusters were obtained whereby the bare silica column was found to be very dissimilar from the other columns. For example, the retention of benzyltrimethylammonium cation was 17.0 min on bare silica but 3.4-12.8 min on the polar bonded packings. Benzyltrimethylammonium cation does not possess any polar functionality except the permanent positively charged ammonium group. On bare silica strong electrostatic attractive interactions occurring between the cationic moiety and dissociated surface silanols may likely the dominant factor for retention. On the other hand, the exceptional low retention of benzyltrimethylammonium cation on non-charged bonded Luna HILIC (t_R = 3.4 min) indicates not only that hydrophilic interactions are largely absent on this packing but also that its cross-linking phase chemistry [43] is an effective shielding to prevent background silanophilic interactions. Such an interpretation agrees well with the retention trend of the quaternary amine thiamine on a larger set of non-charged bonded packings, which could be correlated with the ´silanol activity at pH 7´ of these phases measured in the RP elution mode [42]. Opposed to benzyltrimethylammonium cation the weak base tyramine was considerably stronger retained on Luna HILIC (t_R = 10.5 min) which is explained by a hydrophilic interaction retention increment superimposing the (weak) silanol-solute interactions on this bonded phase. Due to the fact that on bare silica retention times of these two analytes differed by just 0.6 min a pronounced effect of the bonding on chromatographic selectivity was revealed as well.

Opposed to the retention trend of basic solutes, 4-hydroxybenzenesulfonic acid and 4-

hydroxybenzoic acid both eluted earlier on bare silica than on the bonded packings. This finding was ascribed to the presence of a larger degree of negative charge on bare silica which causes ion exclusion, i.e. repulsion, and as a consequence antagonism to hydrophilic interactions. Overall, for both cationic and anionic solutes strong electrostatic forces seem to be a prime factor in determining the extent of retention on bare silica under ´typical´ HILIC elution conditions.

The most similar columns as delivered by HCA were TSKGel Amide-80 and Polysulfoethyl A. This finding is somehow unexpected as TSKGel Amide-80 is considered to be a non-charged bonded polar phase while Polysulfoethyl A is a hydrophilic strong cation exchanger, although with a non-specified density of $R\text{-}SO_3^-$ groups. However, in accordance with earlier interpretations [14,44-46] also the present results indicate that TSKGel Amide-80 has a certain net acidic character (probably stemming from the ligand bonding approach). Ionic forces may thus complement the hydrophilic interaction separation mechanisms provided by the adsorbed water layer and polar ligand functionalities, respectively. A certain relationship of ZIC-HILIC to the two aforementioned packings may be explained by the presence of charged groups in the ligand which may exert strong attractive or repulsive electrostatic interactions with charged analytes. On the other hand, the significant chemometric dissimilarity of these three packings from bare silica demonstrated that a phase with a high degree of available silanols still delivers a different retention profile. This finding points towards the general importance of solute-ligand interactions in the HILIC elution mode (with respect to both strong and weak adsorptive forces) and highlights that a distinct contribution of solute-silanol interactions should not be overlooked in interpreting the retention and selectivity characteristics of bonded phases (*vide infra*).

A distinct grouping from TSKGel Amide-80, Polysulfoethyl A, and ZIC-HILIC was found for the TGO column and Luna HILIC and these two ´diol-type´ phases form a separate cluster. Tyrosine, guanosine, cytidine and tyramine were each 1.5-2.5 min stronger retained on TGO, while benzyltrimethylammonium cation eluted later by more than six minutes. On the other hand, 4-hydroxybenzoic acid was only slightly stronger retained on TGO and for 4-hydroxybenzenesulfonic acid stronger retention was observed on the Luna HILIC column. In case retention on the two phases is mainly determined by polarity-related hydrophilic interactions one would expect the much more polar 4-hydroxybenzenesulfonic acid ($\log D^{pH\ 5\text{-}7}$ = -5.2) to elute considerably later than 4-hydroxybenzoic acid ($\log D^{pH\ 5\text{-}7}$ = 0.85 – -0.94). Ligand-selective retention increments may in part be responsible for the deviation observed on TGO but, overall, the retention behaviour of this strong acid but also of the strong base benzyltrimethylammonium cation is quite plausibly explained by a different background of accessible silanols on TGO and Luna HILIC (earlier characterised to be significantly higher for

TGO [42]). Although the large fraction of ACN as well as the non-adjusted pH of the mobile phase renders an estimation of the degree of ionisation of both silanols and acidic solutes difficult one can assume that at least 4-hydroxybenzenesulfonic acid is almost fully dissociated under the chosen conditions and thus its retention will respond strongly to the presence of residual negative surface charge. Consequently, it appears that a systematic study of retention patterns of charged solutes may deliver descriptors for silanol-solute interactions in the HILIC elution mode, at least for non-charged bonded phases. Parallel work is devoted to this problem and the results will be reported in due time.

At this point, the reader should bear in mind that no data are yet available to which extent a change of elution conditions (e.g. switch to isocratic elution mode; effect of pH, type/amount of salt, type/amount of protic modifier; temperature) will affect the chemometric column classification plotted in Figure 2. Whether the retention profile of the chosen compound mixture is able to adequately reflect the plurality of interactions between solute and adsorbed water layer/chromatographic ligand/sorbent surface is currently unknown as well. However, for the purpose of the present study valuable information was obtained on the different separation properties of the selected columns. Based on this data set it was interesting to study whether the observed column dissimilarities in this targeted approach are reflected in the abilities of the individual columns to differentiate samples by means of chemometric similarity analysis of chromatograms (non-targeted approach).

3.2 Extraction procedure

To allow for a comprehensive characterisation of *Ganoderma* specimens by both HILIC and RP a sample preparation protocol was required which allowed the extraction of compounds of a broad polarity range. A preliminary experimental series with extraction solvents composed of different mixing ratios of ACN and water revealed that the gross peak pattern was not significantly affected when the volume fraction of water was varied within 25-75 %. The final method was developed using a mixture of ACN and water (50:50; *v*/*v*) in a 1:20 *w*/*v* sample-solvent ratio (each 60 min, 50 °C). ACN/water (50:50; *v*/*v*) sample solutions concentrated by a factor of 40 compared to the original extract volume were used for injection. These solutions had higher elution strength compared to the starting gradient conditions in both HILIC (mobile phase consisting of 5 % *v*/*v* water at 0 min) and RP (mobile phase consisting of 10 % *v*/*v* ACN at 0 min). However, the selection of 2 µL as injection volume secured that no significant effects on peak shape or retention time of early eluting solutes occurred as a result of this eluotropic mismatch and the injection volume was already sufficiently high to deliver informative chromatograms in both elution modes.

3.3 Method validation

Validation of the developed HILIC and RP methods focused on assessing the repeatability of the gross peak pattern in terms of RSD values of RRT and RPA for some characteristic peaks. In order to keep the work load in a reasonable range sample no. 14 was selected as a representative for the other specimens and only the ZIC-HILIC column was used for HILIC validation runs. Five (HILIC; t_R-range 4-21 min) and six (RP, t_R-range 14-29 min), respectively, peaks were selected and from each peak no. 4 ($t_R^{ZIC\text{-}HILIC}$ = 12.5 min, t_R^{RP} = 18.0 min) RRTs and RPAs were calculated for the other peaks. Injection precision (n = 5), intra-day precision (n = 5), and 48 h autosampler tray stability were investigated. Tables 3 and 4 summarise the obtained data.

<Table 3>

<Table 4>

Injection precision (n = 5) was found to be ≤ 1.78 %RSD (HILIC) and ≤ 0.79 %RSD (RP) for RRT (Table 3). Corresponding values for RPA were maximal 5.79 %RSD (HILIC) and 2.65 %RSD (RP), thus indicating a slightly lower run-to-run repeatability for HILIC in terms of RRT and RPA. Intra-day precision (n = 5) values were for both elution modes in a similar range for both RRT (0.23-1.95 %RSD for HILIC, 0.43-0.69 %RSD for RP) and RPA (2.78-6.95 %RSD for HILIC, 3.43-6.78 %RSD for RP) and an acceptable repeatability of the sample extraction procedure was thereby confirmed. Stability testing of post-extraction sample solutions was carried out within a 48 hrs period in which a processed solution of sample no. 14 was injected repetitively while stored in the autosampler. RSD values of RRTs below 2 % and below 5 % for RPAs were not significantly different from the figures obtained for the aforementioned injection precision and intra-day precision and thus acceptable chemical stability of the selected compound peaks was assumed. Overall, the experimental data suggested that the developed extraction procedure and the LC methods were applicable for fingerprint analysis of *Ganoderma* specimens.

3.4 *Ganoderma* species discrimination by chromatographic fingerprinting

The analysis of *G. lucidum* samples in the HILIC elution mode delivered quite different peak patterns on the various polar packings (Figure 3). This finding corroborates the above described column-specific separation properties.

<Figure 3>

Over the 45 min time scale of the binary gradient the chromatograms of each HILIC phase contained a number of peaks which were suitable for chemometric fingerprint analysis. However, all chromatograms were also characterised by a bulk of peaks eluting close to the void volume. This was indicative for the presence of less polar compounds in the sample extracts for which the HILIC elution mode did not provide sufficient retention and selectivity. Such poorly separated peaks cannot be used for chromatographic fingerprinting. Quality control or sample discrimination solely based on HILIC data may thus leave out information about less hydrophilic compounds and, consequently, samples which mainly differ by their composition in less to non-polar solutes may not be adequately characterised by such a single-mode approach. On the other hand, also the RP method was of limited selectivity as demonstrated by a large peak eluting at the void time (Figure 3g). Thus, a combined analysis of the samples in HILIC and RP elution modes was regarded appealing to broaden the accessible compound polarity window in order to end up with more discriminative information for *Ganoderma* sample classification.

Chromatograms of *G. lucidum* samples no. 1-11 which were collected from some different locations in China (cf. Table 2) were used to construct column-specific simulative mean chromatograms. Figure 4 exemplarily shows measured and calculated chromatograms as obtained on the ZIC-HILIC column and the RP packing.

<Figure 4>

Correlation coefficients of each experimental chromatogram to the reference chromatogram were calculated according to eq. (1) as detailed in section 2.2.5. With the exception of the bare silica column, correlation coefficients were better than 0.80, typically better than 0.90. Variations of correlation coefficients in terms of %RSD values were 1.3-4.3, while the bare silica column delivered 21.4 %RSD (range 0.36-0.88).

It might be argued that the exceptional high variation on bare silica originated from (cationic) sample extract constituents being accumulated on the non-modified silica surface. Such compounds may then either elute in subsequent runs thereby adulterating peak profiles of later injected extracts or, if being irreversibly adsorbed on the surface, they may change the separation properties of the column itself. Supporting evidence for the latter comes from the fact after less than thirty runs of real samples retention times of some of the test compounds on the bare silica column differed by more than 10 % to the original values while such a large deviation was not observed for the bonded phases.

To validate the species selectivity of the developed reference chromatograms two wild *G. lucidum* specimens (both independent from the training set) as well as ten samples stemming

from *G. sinense*, *G. atrum*, and *G. tsuage* were measured as well (validation set). As depicted in Figure 5 for Luna HILIC and the RP column, the extracts of the various *Ganoderma* species gave to some extent different peak patterns. A certain capability for species discrimination by chemometric similarity analysis of chromatograms was therefore anticipated. For this purpose, the lowest correlation coefficient obtained from the *G. lucidum* training set was set as a column-specific cut-off and samples from the validation set having a correlation coefficient to the calculated reference chromatogram below this cut-off were classified as being "different" from *G. lucidum*.

<Figure 5>

No capabilities to differentiate *G. lucidum* from other *Ganoderma* species could be recognised for the bare silica column, basically due to the aforementioned wide range of correlation coefficients already obtained for the training set (Figure 6a). On the other hand, a successful discrimination of the four samples of *G. atrum* and the one *G. tsuage* specimen from *G. lucidum* could be achieved on all bonded packings. Additionally, the two independent *G. lucidum* samples were correctly classified because the calculated correlation coefficients were each higher than that of the column-specific cut-off values.

<Figure 6>

Some limitations were, however, found for *G. sinense* samples, for which correlation coefficients were, except of the Luna HILIC column, higher than that of *G. atrum* and *G. tsuage*. Specifically, *G. sinense* sample no. 18 was wrongly classified as *G. lucidum* by all HILIC fingerprints (Figure 6). For TSKGel Amide-80 and Polysulfoethyl A results were even worse as each four out of the five *G. sinense* specimens could not be distinguished from the *G. lucidum* reference. This similar behaviour of the two latter columns is somehow a reflection of their chromatographic relationship as has been determined by HCA (*vide supra*). However, overall the HILIC data demonstrated that the type of polar bonded stationary phase had only a minor effect on the capability to discriminate *Ganoderma* samples by means of chromatographic fingerprinting. In other words, a change of the column in HILIC, although potentially leading to quite different peak patterns (cf. Figure 3), was insufficient to avoid sample misclassification, probably owing to limited orthogonality of the chosen polar packings. Complementary analysis of the *Ganoderma* sample extracts in the RP elution mode on the high-efficiency C_{18} packing allowed a differentiation of *G. tsuage* and three out of four *G. atrum* samples from *G. lucidum* (Figure 6g) and the two independent *G. lucidum* samples were correctly identified as well. Sample no. 19 had the highest correlation coefficient within the set

of *G. sinense* samples and it was wrongly assigned to be *G. lucidum*. Interestingly, this sample had lowest correlation coefficients on all HILIC columns which indicated that sample no. 19 differs from *G. lucidum* basically by its composition in hydrophilic compounds.

Lumping together these results unveils some limitations of the single-mode chromatographic fingerprint approaches: (i) When using the bare silica column it was not at all possible to differentiate *G. lucidum* from other *Ganoderma* species, (ii) *G. sinense* sample no. 18 was misclassified by all polar bonded phases in the HILIC elution mode, (iii) *G. sinense* samples no. 12, 13, 14 were misclassified by the HILIC fingerprints when using TSKGel Amide-80 or Polysulfoethyl A, (iv) samples no. 19 (*G. sinense*) and 20 (*G. atrum*) were misclassified in the RP elution mode.

In an attempt to decrease the rate of misclassification observed in the single-mode approaches one may now combine HILIC and RP data. In such a multi-modal concept only samples are assigned being *G. lucidum* when the correlation coefficients in HILIC and RP are each above the cut-off values in the respective chromatographic mode. Following this idea a 100 % correct sample discrimination rate was obtained for the present sample set because the critical *G. sinense* specimen no. 18 (misclassification on all polar columns) was correctly classified by the RP fingerprint. Moreover, the *G. sinense* samples which were misclassified on TSKGel Amide-80 and Polysulfoethyl A were correctly differentiated from *G. lucidum* in the RP elution mode. *Vice versa*, *G. sinense* sample no. 19 and the *G. atrum* sample no. 20, which were both assigned to be *G. lucidum* in the RP elution mode, were correctly classified being different from *G. lucidum* by the chromatographic results of all bonded HILIC phases. Thus, irrespectively of the type of polar bonded stationary phase sufficiently orthogonal information to the HILIC fingerprint was always obtained when RP data were additionally taken into account.

From a biological point of view, the results of the chemometric similarity analysis indicated a closer relationship of the chemical composition of *G. lucidum* and *G. sinense* compared to that of *G. lucidum* and *G. atrum* as well as to that of *G. lucidum* and *G. tsugae*, at least with regards to the compounds accessible by the selected LC-UV conditions. However, the remarkable scatter of correlation coefficients obtained for *G. sinense* and *G. atrum* samples (cf. Figure 6) demonstrated the need for analysing a larger number of specimens in order to cover within-species variation more adequately and, consequently, to obtain more valid criteria for species discrimination when it comes to routine use.

4 Conclusions

Two prime parameters that potentially affect the information content of chromatographic fingerprints were investigated in the present study, *viz.* ´type of stationary phase´ and ´chromatographic mode´.

The chromatograms obtained on the various polar bonded packings were largely similar in terms of their usability for chemometric discrimination of some *Ganoderma* species from *G. lucidum*. In contrast, bare silica turned out to be unsuitable for analysing such complex biological samples under the selected elution conditions. The peak patterns of the real samples differed remarkably between the different polar columns. These findings, along with the column classification based on HCA, indicated a certain degree of orthogonality, which, however, did not strongly affect the fingerprint-based sample discrimination capability. Most samples were correctly classified already in the HILIC elution mode while the additional consideration of RP data was a viable means to end up with a 100 % correct sample classification rate in the present application. Thus, instead of using different columns in one chromatographic mode multi-modal fingerprinting in largely orthogonal chromatographic modes is regarded an attractive concept for reaching more comprehensive and objective information about sample composition for the purpose of identification, characterisation, or quality control of TCM-relevant samples and complex biological specimens in general.

As concerns the still barely understood mechanisms that generate retention in the HILIC elution mode the data obtained for the small set of charged and non-charged test compounds provided valuable preliminary information. The results allowed concluding that besides ´hydrophilic interactions´ (irrespectively if being based on partitioning of compounds between mobile phase and water-enriched surface layer or weak electrostatic solute-ligand/solute-surface adsorption) also strong electrostatic interactions between charged entities (solute-ligand, solute-silanol) are often of relevance and they may act synergistically (ion exchange/attraction) or antagonistically (ion exclusion/repulsion) to the hydrophilic interaction mechanism. It is hoped that future work will allow gaining deeper insight in the processes being actually at work in so-called HILIC separations in order to exploit more straightforwardly and comprehensively this promising chromatographic mode.

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Figure legends

Figure 1 Structural formulas of the compounds used for column classification in the HILIC elution mode.

Figure 2 Classification of polar column packings by hierarchical cluster analysis (HCA). Retention times of the analyte test set (cf. Figure 1) were used as input parameters. Chromatographic conditions are specified in the Materials and Methods section.

Figure 3 Separation of *G. lucidum* sample no. 3 on the set of stationary phases investigated in the present study: (a) Daisogel, (b) TGO, (c) Luna HILIC, (d) TSKGel Amide-80, (e) Polysulfoethyl A, (f) ZIC-HILIC, (g) C_{18} packing. Chromatographic conditions are specified in the Materials and Methods section.

Figure 4 Individual chromatograms of *G. lucidum* specimens (training set, i.e. samples no. 1-11) and calculated reference chromatogram as calculated by computer-aided similarity evaluation: (a) HILIC elution mode (ZIC-HILIC column), (b) RP elution mode. Chromatographic conditions are specified in the Materials and Methods section. Dashed lines indicate peaks matched by the software.

Figure 5 Chromatograms of sample extracts of *G. lucidum* (samples no. 10 and 21), *G. sinense* (sample no. 18), *G. atrum* (sample no. 15), and *G. tsuage* (sample no. 23) obtained in the (a) HILIC elution mode (Luna HILIC column) and (b) RP elution mode. r = correlation coefficient with respect to the reference chromatogram calculated on the basis of the *G. lucidum* training set chromatograms. All chromatograms are base-peak normalised. Chromatographic conditions are specified in the Materials and Methods section.

Figure 6 Scatter plots of column-specific correlation coefficients (i.e. similarity of measured chromatograms to the calculated reference chromatogram of the training set) used for species discrimination: (a) Daisogel, (b) TGO, (c) Luna HILIC, (d) TSKGel Amide-80, (e) Polysulfoethyl A, (f) ZIC-HILIC, (g) C_{18} packing. Solid lines represent the mean correlation coefficient for each *Ganoderma* species and dashed lines represent the value of the lowest correlation coefficient within the *G. lucidum* training set. Points lying below the dashed lines were classified as being “different” from *G. lucidum*.

Figure 1

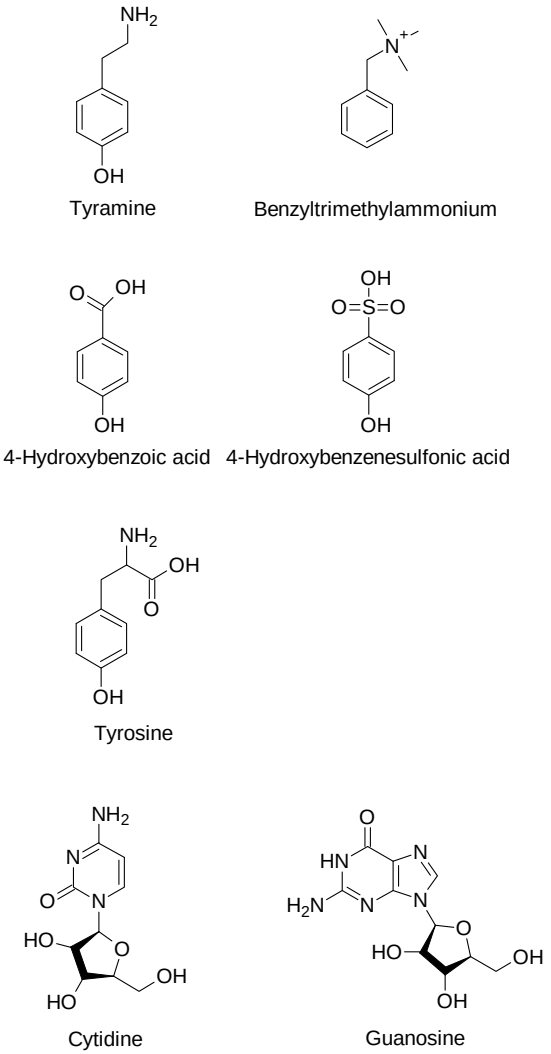

Figure 2

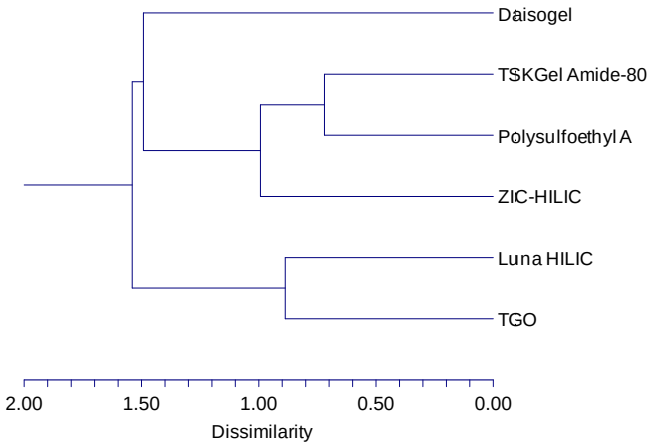

Figure 3

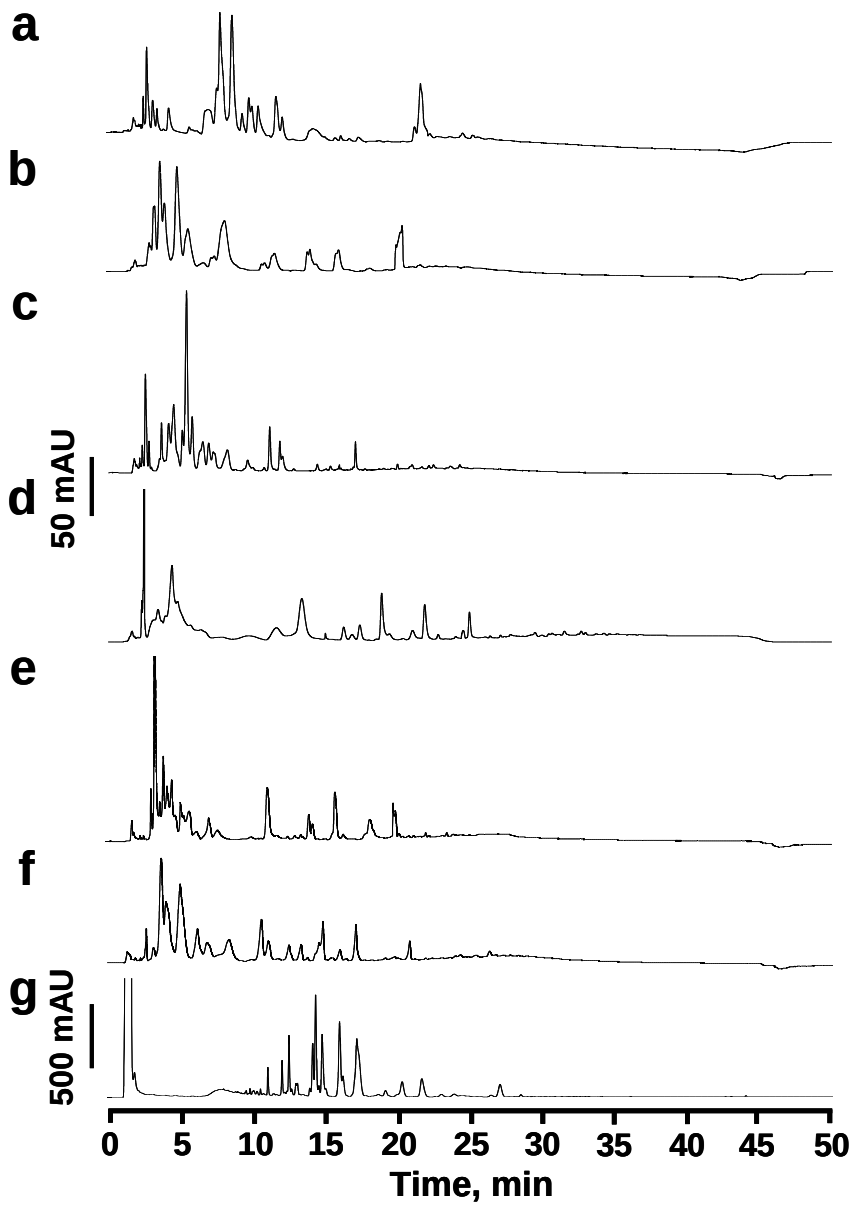

Figure 4

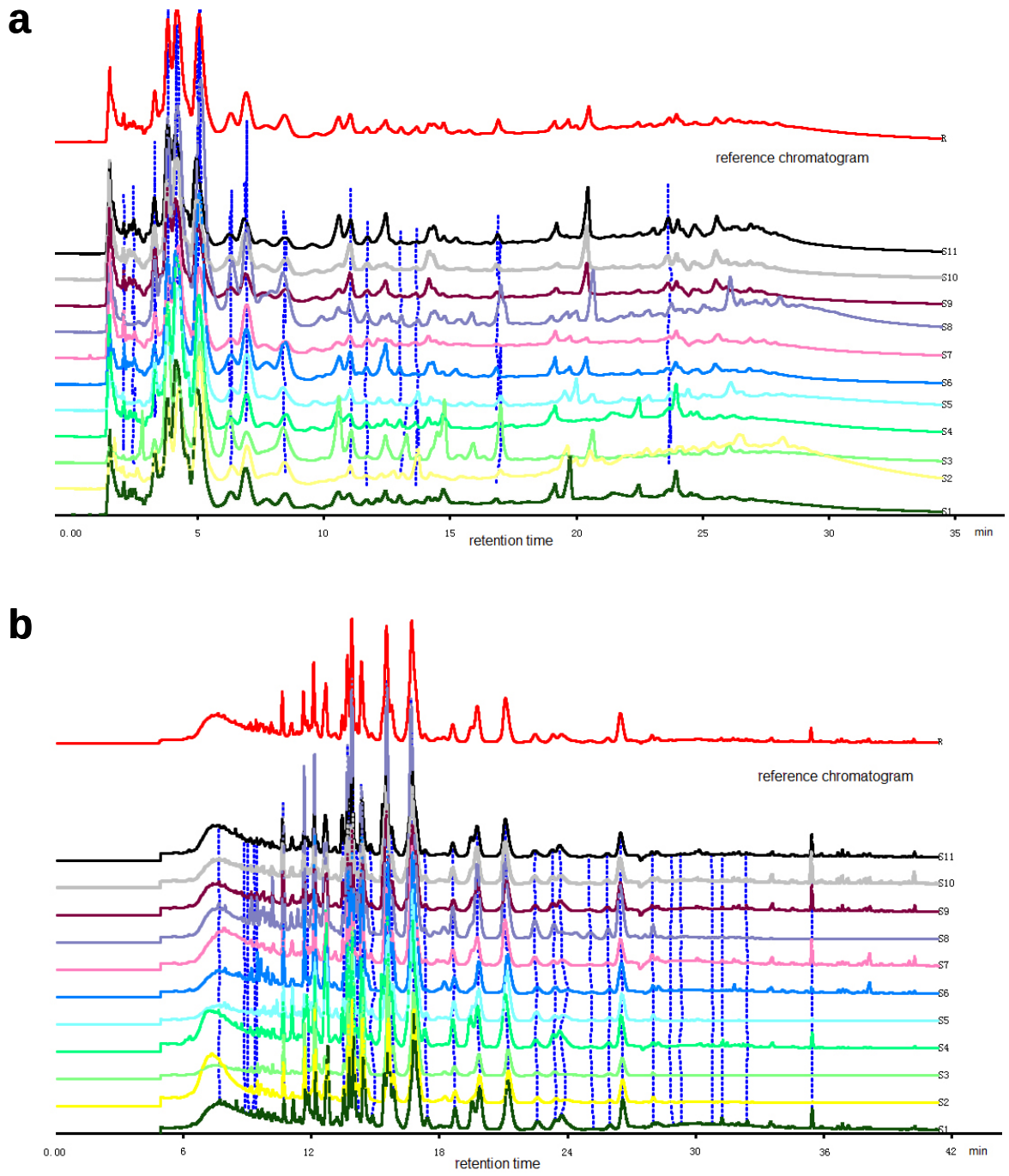
a
R
reference chromatogram
S11
S10
S9
S8
S7
S6
S5
S4
S3
S2
S1
0. 00
5
10
15
retention time
20
25
30
35
min
b
R
reference chromatogram
S11
S10
S9
S8
S7
S6
S5
S4
S3
S2
S1
0. 00
6
12
18
retention time
24
30
36
42
min

Figure 5

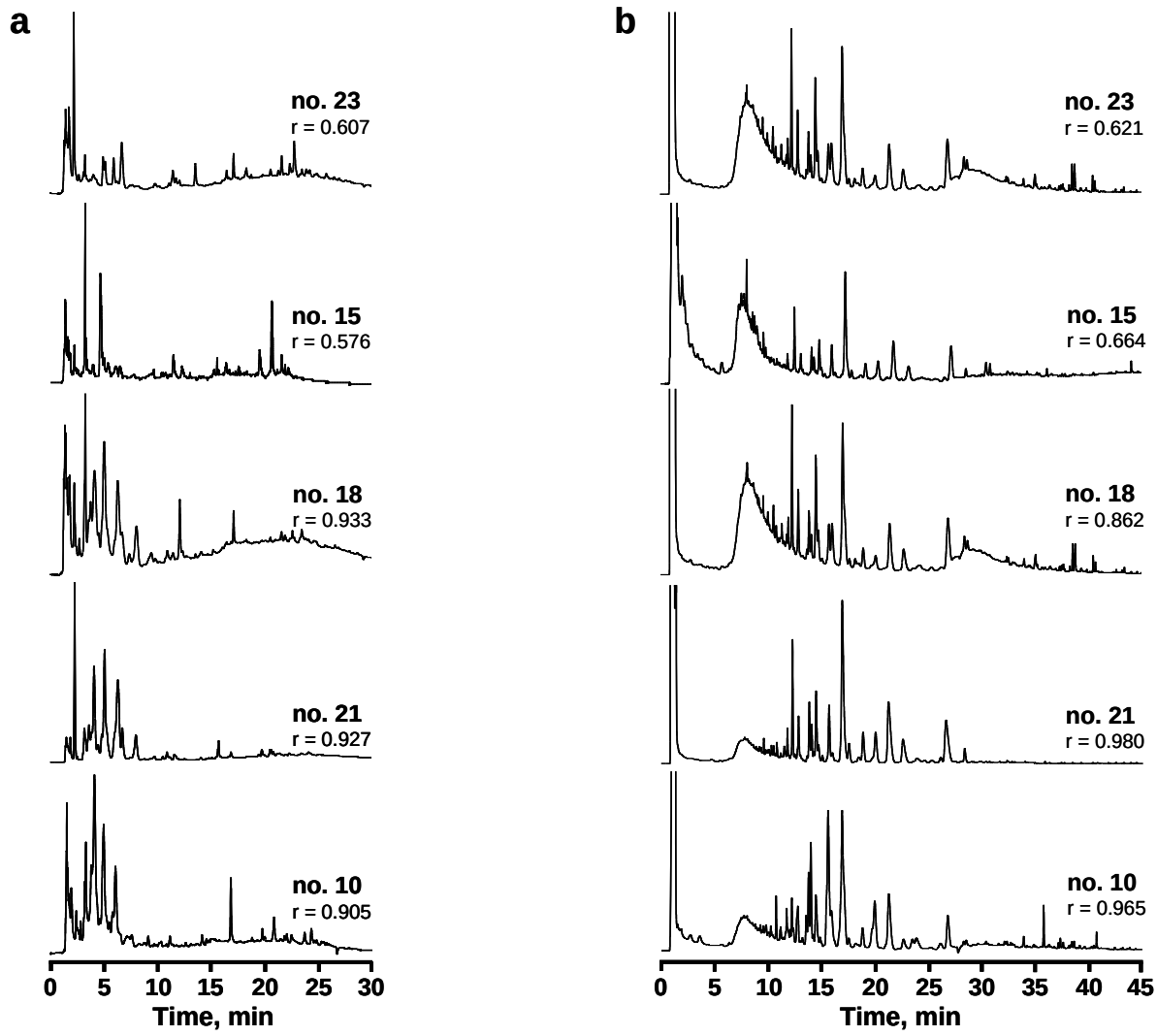
a
no. 23
r = 0.607
no. 15
r = 0.576
no. 18
r = 0.933
no. 21
r = 0.927
no. 10
r = 0.905
0 5 10 15 20 25 30
Time, min
b
no. 23
r = 0.621
no. 15
r = 0.664
no. 18
r = 0.862
no. 21
r = 0.980
no. 10
r = 0.965
0 5 10 15 20 25 30 35 40 45
Time, min

Figure 6

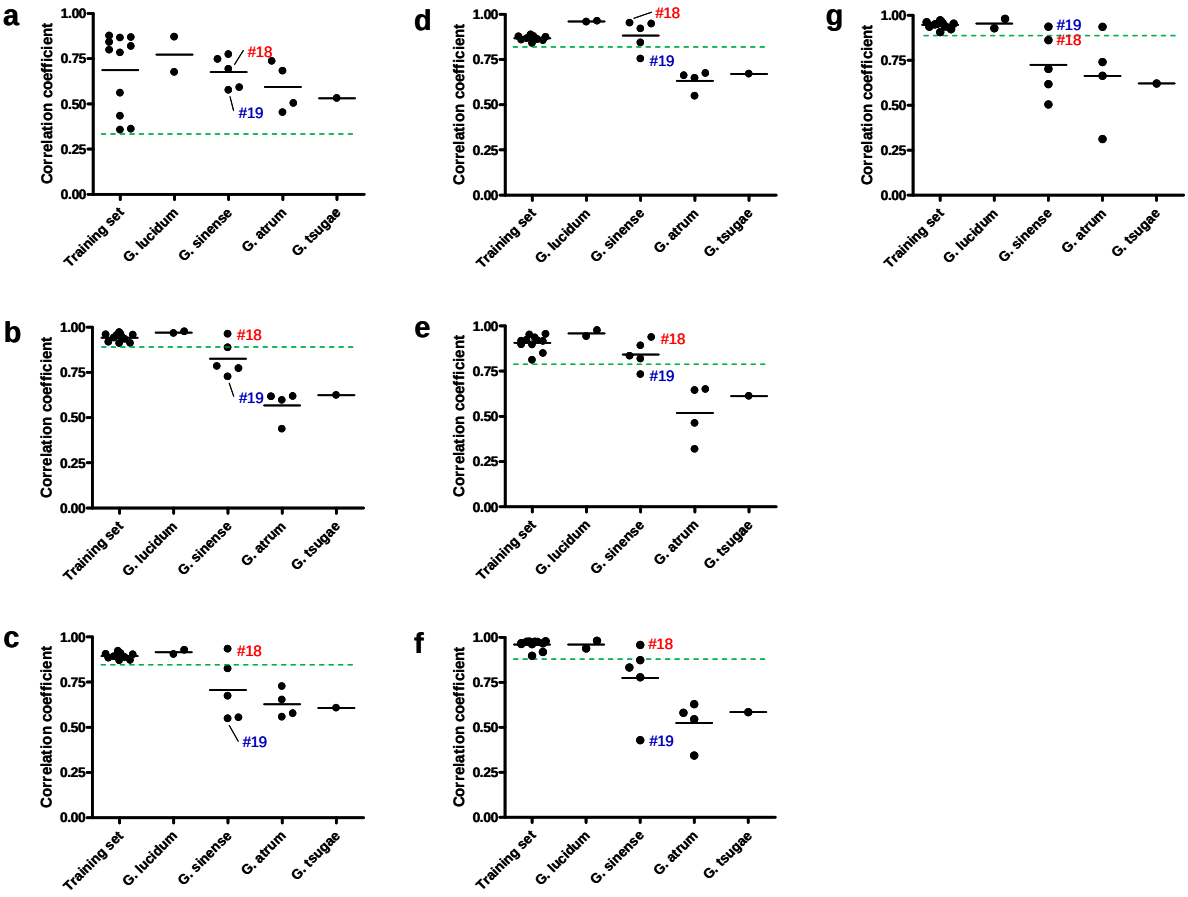
a
b
c
d
e
f
g
Correlation coefficient
1.00
0.75
0.50
0.25
0.00
#18
#19
Training set
G. lucidum
G. sinense
G. atrum
G. tsugae

Table 1 Physico-chemical properties of the evaluated silica-based polar column packings.

Brand name	Particle size [µm]	Pore size [Å]	Polar bonding[a]
Daisogel	5	120	- (bare)
TGO (home-made)	5	120	C_5 ligand with sulfoxide and diol group
Luna HILIC	5	200	Ethylene-bridged diol ligand
TSKGel Amide-80	5	80	Carbamoyl-type ligand
ZIC-HILIC	5	200	Sulfobetaine-type ligand
Polysulfoethyl A	5	300	Poly(2-sulfoethyl aspartamide) ligand

[a]…according to respective manufacturer, for description of home-made TGO packing see [23,42].

Table 2 *Ganoderma* samples analysed in the present study.

Species	Commercial name (China)	Origin (all China)	Sample No.
Training set			
G. lucidum	Golden Lingzhi	Yinshan, Hubei	8
G. lucidum	Golden Lingzhi	Taishan, Shandong	9, 10
G. lucidum	Lingzhi	Longquan, Zhejiang	4, 7
G. lucidum	Lingzhi No. 6	Huangshan, Anhui	6
G. lucidum	Lingzhi G8	Jinzhai, Anhui	2
G. lucidum	Korean Lingzhi	Jiaxiang, Shandong	1, 5
G. lucidum	Korean Lingzhi	Jingdangpu, Shandong	11
G. lucidum	Taishan Lingzhi	Wangdangpu, Shandong	3
Validation set			
G. lucidum[a]	Lingzhi	Lijiang, Yunnan	21, 22
G. sinense[a]	Zizhi	Jinzhai, Anhui	19
G. sinense[a]	Zizhi	Lijiang, Yunnan	13, 14
G. sinense	Zizhi	Wuyishan, Fujian	12, 18
G. atrum[b]	Bao-zi-fen	Ganzhou, Jiangxi	15
G. atrum	Black Lingzhi	Ganzhou, Jiangxi	16, 17, 20
G. tsugae	Songshan Lingzhi	Wangdangpu, Shandong	23

[a]…wild samples
[b]…broken spores

Table 3 HILIC elution mode precision (injection, intra-day) and 48 h post-preparation sample stability of sample no. 14 (*G. sinsense*).

Peak no.[a]	**Injection precision**[b]		**Intra-day precision**[b]		**48 h stability**[c]	
	%RSD RRT[d]	%RSD RPA[e]	%RSD RRT	%RSD RPA	%RSD RRT	%RSD RPA
1	1.78	4.12	1.95	3.84	0.34	1.79
2	1.32	5.79	0.23	6.95	0.16	3.94
3	0.65	2.36	1.56	2.78	0.38	3.21
5	0.53	2.48	0.67	2.96	0.12	2.05

[a]…Peak no. 4 was used for normalisation

[b]…$n = 5$

[c]…seven time points

[d]…RRT = relative retention time

[e]…RPA = relative peak area

Table 4 RP elution mode precision (injection, intra-day) and 48 h post-preparation sample stability of sample no. 14 (*G. sinsense*).

Peak no.[a]	**Injection precision**[b]		**Intra-day precision**[b]		**48 h stability**[c]	
	%RSD RRT[d]	%RSD RPA[e]	%RSD RRT	%RSD RPA	%RSD RRT	%RSD RPA
1	0.66	1.09	0.68	3.59	1.23	3.65
2	0.51	2.44	0.43	3.43	1.16	1.53
3	0.40	3.1	0.53	5.18	0.73	1.87
5	0.54	2.03	0.56	4.85	0.79	4.76
6	0.79	2.65	0.69	6.78	1.87	4.23

[a]…Peak no. 4 was used for normalisation

[b]…$n = 5$

[c]…seven time points

[d]…RRT = relative retention time

[e]…RPA = relative peak area

ADDENDUM # 5

Manuscript (MS5)

draft

Effect of surface silanols on retention and selectivity properties of non-charged polar packings in hydrophilic interaction chromatography

Junyan Wu, Wolfgang Bicker, Wolfgang Lindner

Christian Doppler Laboratory of Molecular Recognition Materials, Department of analytical Chemistry and Food Chemistry, University of Vienna, Vienna, Austria

1 Introduction

Hydrophilic interaction chromatography (HILIC) is an established liquid chromatographic (LC) mode for the separation of polar solutes. Its current application field spans from pharmaceutical drug impurity profiling to metabolomics/metabonomics. The technical term ´HILIC´ was coined in 1990 by Alpert [1], while the underlying separation concept already dates from the early days of LC [2]. The augmented practical popularity of HILIC within the past few years, well reflected for example in a recent special issue [3], encouraged separation scientists to shed light on the individual contributions of stationary phase and mobile phase parameters to retention and selectivity in order to better control and optimise HILIC-type separations.

The fundamental principle of HILIC is characterised by the differential distribution of hydrophilic solutes between a polar stationary phase and a low-polar mobile phase. The eluent is usually composed of a non-protic organic solvent such as acetonitrile (ACN) which is polar-modified by water in a volume fraction of typically 0.05 to 0.4 to yield proper elution strength. Partial or even complete substitution of the water fraction by polar protic modifiers has recently been proposed as an additional way to adjust retention and to introduce selectivity increments as well [4-6]. The chromatographic process may also be affected by the type and amount of organic salt additives as well as the pH value. Careful optimisation of the mobile phase composition like also of the temperature is therefore central to the development of HILIC-type separations.

The large number of packings that can be operated under HILIC elution conditions extends the toolbox available for method development even more. The basic criterion of a chromatographic material for its HILIC applicability is its polarity. In the simplest manner this is realised by using bare silica [7]. Furthermore, a number of non-charged polar

ligands bonded to silica support turned out to possess advantageous HILIC properties, among them bondings containing amide, hydroxy, or sulfoxide groups [4, 8, 7]. Common as well is the use of silica materials modified with cationic, or anionic, or zwitterionic functionalities [7].

It is a current matter of scientific debate whether the retention under HILIC elution conditions is driven by partitioning of the analytes between a layer of protic modifier dynamically enriched on the polar sorbent surface and the less polar mobile phase or if it is of adsorptive nature, i.e. if direct solute-ligand interactions occur. In this context, interactions between charged solutes and charged ligand functionalities (i.e. ionic forces) have to be clearly differentiated from those involving non-charged polar entities (hydrogen bonding and other dipole-dipole forces). Only the latter are termed “hydrophilic interactions” in a strict sense and they form the basis for retention in HILIC irrespectively if it is primarily based on partitioning or adsorption. Additional adsorptive mechanisms based on ion exchange or ion repulsion can nevertheless contribute valuably to hydrophilic interactions. Such a type of mixed-mode chromatography composed of strong and weak electrostatic contributions to retention is a versatile and promising approach for many polar separation problems [9-11].

Ligand density plays a decisive role in all kinds of chromatography with bonded packings. In silica-based reversed-phase (RP) chromatography this crucial impact comes basically from the overall stationary phase lipophilicity, which results from the degree of alkyl silane surface functionalisation. Silanols remaining non-modified may become involved into the retention process especially with regards to basic solutes [12, 13] and in case of using packings with improper silanol end-capping. Silanophilic interactions are quite orthogonal to the intended *non-polar* separation environment of RP. This may sometimes result in detrimental chromatographic effects (e.g. defective peak shapes due to inadequate elution conditions) but may give access to selectivity increments as well.

On the other hand, HILIC with bonded packings faces a somehow different situation. Here not only the ligand but also the remaining non-modified surface silanol network is capable of developing hydrophilic interactions. At first glance this leads to a more “homogenous” separation environment compared to RP. However, earlier work speculated also on the presence of ionic silanophilic interactions when polar charged solutes were separated on polar-bonded HILIC columns [8, 14, 15]. These may potentially superimpose the hydrophilic interaction retention process or it may even be the dominant factor.

The aim of present work was to study systematically chromatographic effects in terms of

retention and selectivity caused by varying the ligand density and the residual silanol fraction, respectively, of a non-charged polar bonded packing (see Figure 1) operated under typical HILIC elution conditions. The results extended our understanding about retention mechanisms on such types of mobile phases and how these mechanisms can be influenced by ligand surface coverage and elution conditions.

2 Experimental

2.1 Chemicals

HPLC gradient grade acetonitrile (ACN) and ethanol were obtained from VWR International (Vienna, Austria). Water was bi-distilled in-house. Formic acid, ammonium formate, acetic acid, ammonium acetate, ammonium trifluoroacetate, triethylamine and 25 % *v*/*v* aqueous ammonia solution were all of analytical grade and supplied by Sigma-Aldrich (Vienna, Austria). Chromatographic test compounds originated from Sigma-Aldrich (see Figure 2). 1-[3-(trimethoxysilyl)propyl]urea 97% solution was obtained from Sigma-Aldrich (Vienna, Austria) and 4-dimethylaminopyridine originated from Fluka (Buchs, Switzerland). Daisogel (5 µm, 120 Å, 300 $m^2.g^{-1}$) was supplied by Daiso Chemical (Osaka, Japan).

2.2 Preparation of column packings

3 g of bare silica was suspended in 30 mL ethanol and the mixture was reacted at 70°C with 1-[3-(trimethoxysilyl)propyl]urea 97% solution and 4-dimethylaminopyridine (added to the reaction mixture in a molar fraction of 0.05 with regards to the silane) for 19 h under nitrogen. To obtain packings with different ligand surface coverages the silane solution was added to the silica suspension in 0.2, 0.5, and 1.2 $mmol.g^{-1}$ fractions. Another batch of 3g bare silica was suspended in 30 mL ethanol and 2.5 $mmol.g^{-1}$ silane solution was added. The suspension was evaporated in a evaporator until dry. The residue was suspended in 30 mL toluene and the mixture was reacted at 130°C with 4-dimethylaminopyridine for 19h under nitrogen. The modified silica materials were collected by filtration, washed excessively with MeOH, and dried at 60 °C for 24 h *in vacuo*. The proposed ligand structural formula is drawn in figure 2. The mean value of nitrogen load of the packings as determined by combustion elemental analysis was used to calculate ligand densities which

amounted to 0.38, 0.91, 1.81, and 3.67 µmol.m^{-2}. 150 x 4 mm id stainless steel columns were slurry-packed with these materials and bare silica, respectively, using methanol as carrier solvent.

2.3 Preparation of mobile phases

Eluents were composed of ACN and aqueous buffer solutions in a volume ratio of 90 to 10 or 80 to 20. The salt concentration amounted to 3.75 mM, 7.5 mM, 10 mM, 15 mM, or 30 mM of desired salt. pH values were adjusted with acetic acid, formic acid or 25 % *v*/*v* aqueous ammonia solution prior mixing with the required amount to ACN. For detailed mobile phase compositions the reader is referred to the respective Figure legends.

2.4 Chromatographic conditions

HPLC runs were performed on a 1200 series HPLC system from Agilent (Waldbronn, Germany) equipped with a diode array detector. Non UV-detectable solutes (acetylcarnitine) were analysed on a 1100 series HPLC system (Agilent) which was equipped with a Corona charged aerosol detector from ESA Analytical (Aylesbury, UK). All chromatograms were acquired and processed with the HP^{3D} Chemstation software. Test compounds were dissolved in ACN/water (90/10, *v*/*v*) at concentration levels ranging from 0.25 to 1 mg.mL^{-1} and injected in 5 µL fractions. The flow rate was set to 1.00 mL.min^{-1}. The column compartment was kept at 25 °C except where noted. Void times were measured with toluene. Columns were allowed to equilibrate with about 20 column volumes of each new mobile phase.

3 Results

3.1 Separation profiles of bare silica and differently densely bonded packings

A first impression of differences in the retention properties of the bare silica column and the densest bonded packing (3.67 µmol.m^{-2}) was provided by orthogonal plotting of retention factors *k* obtained on these both columns under similar elution conditions for a set of analytes which included bases, acids, zwitterions and neutral compounds (See Figure 2).

Neutral compounds

All investigated neutral analytes except for acetylglucosamine and adenine (group III in Figure 2) under the chosen elution conditions showed stronger retention on the bonded 3.67 µmol.m^{-2} phase as reflected by their position above the 45° equivalence line of the log-log orthogonality plot given by Figure 3. They experienced a gain in retention ranging from 7 to 112 %. A stronger response was delivered for more polar compounds, for example gain in retention factor for guanosine by ca. 64% and for guanine by ca. 41%.

Acidic compounds

Retention of acidic solutes (being part of group I in Figure 2) was found to be more susceptible to the introduction of the ligand onto the silica surface. The chosen analytes exhibited a gain in retention ranging from 34 to 286% when the used stationary phase was switched from bare silica to the bonded packing (3.67 µmol.m^{-2}). On the other hand, substituting the carboxylic group in 4-hydroxybenzoic acid with the stronger acidic sulfonic group resulted in a remarkable decrease of absolute retention on bare silica ($k^{\text{4-hydroxybenzoic acid}}$ = 2.72, $k^{\text{4-hydroxybenzenesulfonic acid}}$ = 0.53) while the gain in retention on the bonded phase was maximal for the stronger acidic solute (gain in k by 286% for 4-hydroxybenzenesulfonic acid and by 34% for 4-hydroxybenzoic acid). It appeared that the structure of analytes had certain effect on retention. It was also noticed that specifically acidic solutes showed the most susceptibility to an increase in phase polarity. This was for example well illustrated by the structural analogues 3,4,5-trihydroxybenzoic acid and 3,4,5-trihydroxybenzamide, the latter being an amide analogue of 3,4,5-trihydroxybenzoic acid with almost no acidic properties. On the bare silica column 3,4,5-trihydroxybenzoic acid was eluted with retention factor k = 5.49 while retention factor was 7.2-fold lower for 3,4,5-trihydroxybenzamide (k = 0,76) thus resulting in a selectivity value α of 6.2. Upon running the same analytes on the densely modified packing 3.67 µmol.m^{-2} selectivity was, however, found to be dropped to 4.85.

Basic compounds and zwitterions

In contrast to the retention pattern of the aforementioned compound classes the basic and zwitterionic compounds (group II in Figure 2) experienced a decrease in retention when the 3.67 µmol.m^{-2} bonded packing instead of the bare silica column was used. This drop extent was especially strong for the charged strong bases (loss in k by ca. 73 % for benzyltrimethyl-ammonium, by ca. 81% for thiamine, by ca. 85 % for acetylcarnitine)

while the loss of retention was by ca. 10% for tyrosine, for melamine by ca. 11%, for 5-hydroxydopamine by ca. 15% and for tyramine by ca. 34%. Thereby, tyrosine showed light fluctuation in retention (k =18.42 on bare silica, 14.23 on 1.81 µmol.m^{-2} and 16.63 on 3.67 µmol.m^{-2} packing). At high surface coverage the accessibility of the surface silanols is suppressed which results in a decrease of attractive ionic forces between the positive charged base and the deprotonated silanol groups.

This data set revealed that the presence of the non-charged polar ligand on the silica sorbent surface has distinct consequences for the retention of non-charged and charged polar analytes. In order to a more detailed look to the actual effect of extent ligand density on retention, other three packings featuring ligand surface coverage of 0.38, 0.91, 1.81 µmol.m^{-2} are also involved to discuss. As given by Figure 4a the gain in k values of neutral (guanine), acidic (4-hydroxybenzoic acid), and the loss in k values of basic compounds (melamine) was clearly linked to ligand density. Reversals of elution order resulting from an increase of ligand density occurred especially for physico-chemically different analytes as illustrated in Figure 4b for neutrals cytidine and guanosine, for acids 4-hydroxybenzenesulfonic acid and cyanuric acid and for bases tyramine and benzyltrimethylammonium (BTMammoniu). Accordingly, selectivity values α were also affected to a certain extent by the ligand surface coverage (see Figure 4b).

The results from this preliminary study clearly showed that depending on the physico-chemical nature of the analyte and the extent of silica surface functionalisation with the chosen non-charged polar motif quite different retention mechanisms seem to be at work. To substantiate this aspect and to closer investigate the differences in the retention mechanisms proposed for neutral, acidic, basic and zwitterionic compounds as a matter of ligand density and residual silanol fraction, respectively, complementary studies aiming at elucidating chromatographic effects resulting from the variation of distinct elution parameters (modifier content, pH value, salt nature and load, column temperature) were carried with the bare silica column and packings having a ligand density of 1.81 and 3.67 µmol.m^{-2}, respectively. Due to the too long retention of acetylcarnitine, in the following study it would be not taken as analyte any more.

3.2 Chromatographic effects of elution parameters as a function of ligand density

3.2.1 Effect of modifier content

Upon decreasing the water fraction in isocratic ACN-based eluents from 20 to 10 % while

keeping the overall ammonium formate concentration constant (10 mM, pH 7) a pronounced increase of retention was observed for all investigated analytes bases, acids, neutrals as well as zwitterions which, however, was ligand density dependent. The retention of the zwitterionic compound tyrosine was the most strongly affected by the aqueous fraction among the chosen solutes and among these three columns the bare silica column gave the strongest response for all solutes except for 4-hydroxybenzenesulfonic acid and guanosine (see figure 5a). Higher ligand density mostly led to a less response to a change of modifier content for basic compounds and zwitterion tyrosine while the response was more scatter for acidic and neutral compounds (see figure 5b). For example, the *k* value of guanine experienced a gain by ca. 175% on bare silica when the eluent contained 10 instead of 20 *v/v* % water while this value was 156% on the 1.81 µmol-m^{-2} and 171% on the 3.67 µmol-m^{-2} bonded packing.

It was noted that the number of phenolic groups present in the benzoic acid motif was found to be one of main factors that determined absolute retention on bare silica and its relative gain upon changing to the bonded packings (eluent contained 10% *v/v* water, bare silica: $k^{\text{4-hydroxybenzoic acid}} = 1.68$, $k^{\text{3,4-dihydroxybenzoic acid}} = 2.34$, $k^{\text{3,4,5-trihydroxybenzoic acid}} = 3.67$). On the other hand, the steric position between phenolic groups (on bare silica: $k^{\text{3,4-dihydroxybenzoic acid}} = 2.34$, $k^{\text{3,5-dihydroxybenzoic acid}} = 2.99$) or between phenolic group and carbonic acid group (eluent contained 10% *v/v* water, on bare silica: $k^{\text{2,3-dihydroxybenzoic acid}} = 0.61$, $k^{\text{2,4-dihydroxybenzoic acid}} = 0.86$) in the benzoic acid motif also play a crucial role in retention (See table 1).

3.2.2 Effect of pH value

As an additional parameter that might affect chromatography in the HILIC mode, two different pH values of the aqueous ammonium acetate fraction were tested (pH 4.0 and 7.0). The mobile phases were ACN/water (90/10, v/v) with 10 mM salt. The bare silica column, 1.81 and 3.67 µmol.m^{-2} bonded packing were comparatively investigated for this purpose.

Retention factors of basic solutes varied less than 10 % between the two investigated pH values for all three packings while the variation of *k* was less than 5% for neutrals and zwitterionic compound tyrosine on both bonded packings and a stronger variation from 10 to 17% on bare silica.

For most acidic compounds the effect of pH value on retention factor was less than 18% upon change the pH value between 4 and 7 on all three investigated packings. But four benzoic acid analogues showed high susceptibility to the changing of pH value (on 1.81

µmol.m^{-2} packing: for 3,5-dihydroxybenzoic acid loss in *k* by 50% varying pH value from 7 to 4, for 3,4,5-trihydroxybenzoic acid by 98%, for 3,4-dihydroxybenzoic acid by 126% and for 4-hydroxybenzoic acid by 128%). These four benzoic acid analogues possess pK_a values from 3.96 to 4.57. At pH value 7 solutes were almost fully dissociated while at pH 4 they were about 50% dissociated. The higher dissociation grad led to higher polarity of solutes, resulting in stronger hydrophilic interaction retention. The buffer pH value can affect the ionisation conditions of solutes and residual silanol groups as well and thus the effect on retention is depending on the type of analytes and the ligand density.

3.2.3 Effect of nature and amount of salt

To draw a rough picture on the chromatographic effects resulting from varying the ammonium acetate concentration of the mobile phase as a function of stationary phase ligand density (0, 1.81 and 3.67 µmol.m^{-2}) eluents composed of ACN/buffer pH 6.0 (90/10, *v*/*v*) containing 3.5 mM, 7.5 mM, 15mM and 30 mM ammonium acetate were tested.

Two opposite trends in changes of retention time upon increasing salt concentration were observed. All investigated acidic and non-charged solutes (4-hydroxybenezenesulfonic acid, cyanuric acid, cytidine and guanosine) experienced an increase of retention at higher salt loads in the eluent on all investigated columns and a stronger response was found for acids. The increase in retention with increasing salt concentration was in line with a result of hydrophilic interactions driven retention mechanism and the higher susceptibility of acids compared to non-charged solutes to the amount of salt was possibly owing to a reduction of electrostatic repulsions between silanol groups and acids at higher salt concentration.

The effects on retention time of bases were more complex. The strong base BTMammonium showed a loss in retention at higher salt concentration (Figure 6), while weak base like melamine exhibited an increase in retention on all columns. Furhtermore, on bare silica column a stronger decrease in retention of BTMammonium was observed than on propylurea-bonded silica columns. The loss in retention of strong bases can be explained by an attractive ‘ionic interactions’ between solute and ionized silanol groups. At higher salt concentration the ammonium ions block the silanol sites and the ionic attraction between strong base and dissociated silanols is partly hampered which led to a weaker retention time of BTMammonium. Bare silica possesses the most amounts of silanol groups on the surface among the used three stationary phases, leading to the strongest shielding effect of silanols which will be present with the most extent of effect of

salt concentration. This aspect was proved by our data (for BTMammonium: loss in *k* by ca. 76% on bare silica and by ca. 36% on the 3.67 µmol-m^{-2} bonded packing). This phenomenon reflects actually the silanophilic interactions. For weak bases, the ionic attractions with ionized silanols were probably not as strong as the hydrophilic interactions which mean in case of weak bases the hydrophilic interactions were primarily responsible for the retention. Thus, the weak base melamine reacted with an increase in retention upon increasing salt concentration as observed for acidic and non-charged solutes. Overall, salt concentration may affect not only on the hydrophilic interactions but also on the solute-silanol interactions and the effect is strongly depending on the type of analytes and the ligand density of bonded columns.

The effect of the nature of salt on retention was investigated with different salts, namely ammonium acetate (NH_4Ac), ammonium formate (NH_4FA), ammonium trifluoroacetate (NH_4F_3Ac) and triethylamine (TEA). Buffers with the same ${}^{w}_{w}$pH value (pH value adjusted in aqueous phase) containing different salt additives can induce different eluent ${}^{s}_{w}$pH values (measured pH value after addition of ACN). The different various ${}^{s}_{w}$pH values of the resulting eluents obtained with ACN/buffer ${}^{w}_{w}$pH 5 (90/10, v/v) are given in the following: NH_4Ac, ${}^{s}_{w}$pH~7.74, NH_4FA, ${}^{s}_{w}$pH~7.2, NH_4F_3Ac, ${}^{s}_{w}$pH~5.13 and TEA, ${}^{s}_{w}$pH~7.59. The different ${}^{s}_{w}$pH values can act on the ionisation conditions of silanol groups on the surface of silica. For example, the BTMammonium was eluted earliest with eluent containing NH_4F_3Ac salt (k = 1.35) on bare silica and the retention factors with mobile phases resulting from other salts were much higher (k = 8.22 with NH_4Ac, k = 7.11 with TEA and k = 6.41 with NH_4FA). But, for strong acids the strongest retention was delivered with NH_4F_3Ac salt. At the eluent ${}^{s}_{w}$pH~5.13 value delivered with NH_4F_3Ac salt, the most silanol groups were probably in associated conditions. Thus, the ionic attractions between strong bases and silanols were suppressed leading to a lower retention while the ionic repulsions between strong acids and silanols were promoted resulting in a stronger retention time. This effect was weaker on bonded columns than on bare silica column. Definitely, the nature of salt is an effective tool to optimise retention and selectivity of polar analytes in the HILIC elution mode.

3.2.4 Effect of temperature

The evaluation of effects of the column temperature on retention was investigated from

15°C to 55°C on the bare silica, 1.81, and 3.67 µmol.m^{-2} propylurea-bonded columns. When the temperature was increased from 15°C to 55°C, all non-charged analytes showed a decrease in retention under elution with eluent ACN/ buffer pH 5 (90/10, v/v) containing 10 mM NH_4Ac on all investigated columns. The situations for basic solutes were more complicated. For strong base BTMammonium, the retention was consistently increased upon an increase of temperature on all columns (Figure 7) which reflected an adsorptive retention mechanism and in case of this, probably the attractive ionic interactions between solute and residual silanols. For the weak bases melamine and tyramine, a gain in retention was delivered on bare silica column and on lowly loaded propylurea-bonded column at higher temperature while a loss in retention was presented on densely bonded silica. It seems that for certain basic solutes the ligand density play a very important role to the retention mechanism which may change the main retention interactions from adsorptive one at low ligand density to hydrophilic one at high ligand density.

4 Discussion

The chromatographic ligand chosen for present study, namely 1-[3-(trimethoxysilyl)-propyl]urea (Figure 1), featured one very hydrophilic urea group which is capable of serving as hydrogen bond donor and/or acceptor. It was therefore assumed that multiple adsorptive hydrophilic interactions with this ligand attached to silica surface can easily be developed by analytes and mobile phase components, respectively. Covalent immobilisation of this polar silane onto silica surface will cause an increase in polarity of the resultant bonded packing. It was therefore assumed that the extent of hydrophilic interactions will change as a function of ligand surface coverage.

Bonding of the selected ligand to the silica surface introduces polar functionalities which in turn will participate in the development of adsorptive hydrophilic interactions between (solvated) solute and (solvated) ligand and the (solvated) silica surface, respectively. If, however, these hydrophilic interactions occur predominantly with water present in the mobile phase a more polar packing will build up a thicker water layer thus leading to a difference in the phase ratio compared to the less polar material (bare silica). Consequently, solutes being subjected to a partitioning process will experience stronger retention in case of an increased layer of dynamically absorbed water. This aspect was

noticed in our date. For example, 4-hydroxybenzenesulfonic acid delivered retention factor k = 0.53 on bare silica material, 0.95 on 1.81 µmol.m^{-2} and 2.04 on 3.67 µmol.m^{-2} columns with mobile phase ACN/H_2O (90/10, *v/v*) containing 10mM ammonium acetate with pH 6.

As above showed, in this study used chromatographic ligand is a non-charged hydrophilic functionality. The absence of any chargeable functionality in the ligand structure prevented the occurrence of attractive or repulsive ionic solute-ligand interactions which in case of charged solutes may easily become the dominant factor in the retention mechanism despite using HILIC-typical elution conditions. Nevertheless, if indications for ionic interaction increments would be found (*e.g.* the negative effect of increased salt content on retention for charged solute benzyltrimethyl-ammonium, in *3.2.3* section) these could safely be ascribed to dissociated silanols being present at the sorbent surface and getting involved in the retention process. Furthermore, the observed decrease in retention on the more polar packing as observed for charged analytes was in sharp contrast to what is to be expected for a hydrophilic interaction type retention mechanism. This strongly indicated fundamental differences in the retention mechanisms of acidic and basic solutes.

For solutes being non-charged under the chosen elution conditions the column-dependent retention pattern can readily be explained by differences in the polarity of the stationary phase and consequences arising for the extent for hydrophilic interactions. For such a scenario it can also be expected that retention of more polar non-charged solutes will respond more to an increase in stationary phase polarity and this was confirmed by the experimental results.

However, changes in the retention of charged solutes fail to be adequately explained by a “pure” hydrophilic interaction retention mechanism. Yet, both of these phenomena for acids and for bases can be readily explained if ionic interactions between dissociated silanols and charged solutes are considered. Under the chosen pH 6 value of the mobile phase a considerable fraction of surface silanols will be available for ionic interactions. These will also not be fully suppressed by ion pairing of NH_4^+ present in the eluent (10 mM ammonium acetate). As a consequence acidic solutes experience repulsion on the bare silica column to a high degree which antagonises attractive hydrophilic interactions to a certain extent. Upon changing to a packing with a lower fraction of surface silanols, i.e. a polar bonded one, retention increases due to a decrease of repulsive ionic forces and maybe also due to an increase in hydrophilic interactive sites. Basic solutes, in opposite to the behaviour of acidic solutes, displayed a decrease in retention because of the loss of their primary attractive interaction sites upon bonding the non-charged ligand to the silica

surface. In this case the hydrophilic interactions do not play a significant role to the retention of this set of bases, at least not major role.

5 Conclusion

Two opposite effects on retention for a set of acidic, neutral, basic and zwitterionic solutes were observed when the 1-[3-(trimethoxysilyl)propyl]urea ligand density on the surface of silica was varied from 0 to 3.67 μmol.m^{-2} i.e. varying the residual silanol fraction. For the investigated acid and non-charged compounds the retention factor increased in an extent from 16% to 286% with an increase of ligand density from 0 to 3.67 μmol.m^{-2} while for bases and zwitterions a decrease of retention from 10% to 85% was present. The drop of retention was strongest responded for charged compounds (for BTMammonium loss in *k* by ca. 73%, for melamine by ca. 11%). Consequently selectivity was affected in certain extent, for example $\alpha^{\text{3,4,5-trihydroxybenzoic acid/3,4,5-trihydroxybenzamide}}$ value was 6.2 on bare silica and value was 4.85 on 3.67 μmol.m^{-2} packing). The fundamental cause of this phenomenon was the differences in retention mechanism on columns. All investigated variables of mobile phase, namely the content of modifier, the buffer pH value, the nature and amount of salt and the column temperature were effective to optimise retention and selectivity in HILIC mode and the extent of responses was strongly depending on the type and the nature of analytes and the ligand density of silica phase as well. A mixed-mode retention process composed of partition hydrophilic interaction and adsorptive interaction such as hydrogen bonding between solutes and ligands and attractive or repulsive ionic interaction between solutes and residual silanol groups is a reasonable understanding.

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Figure legends

Figure 1 The structural formula of the used chromatographic ligand

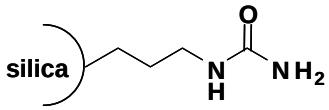
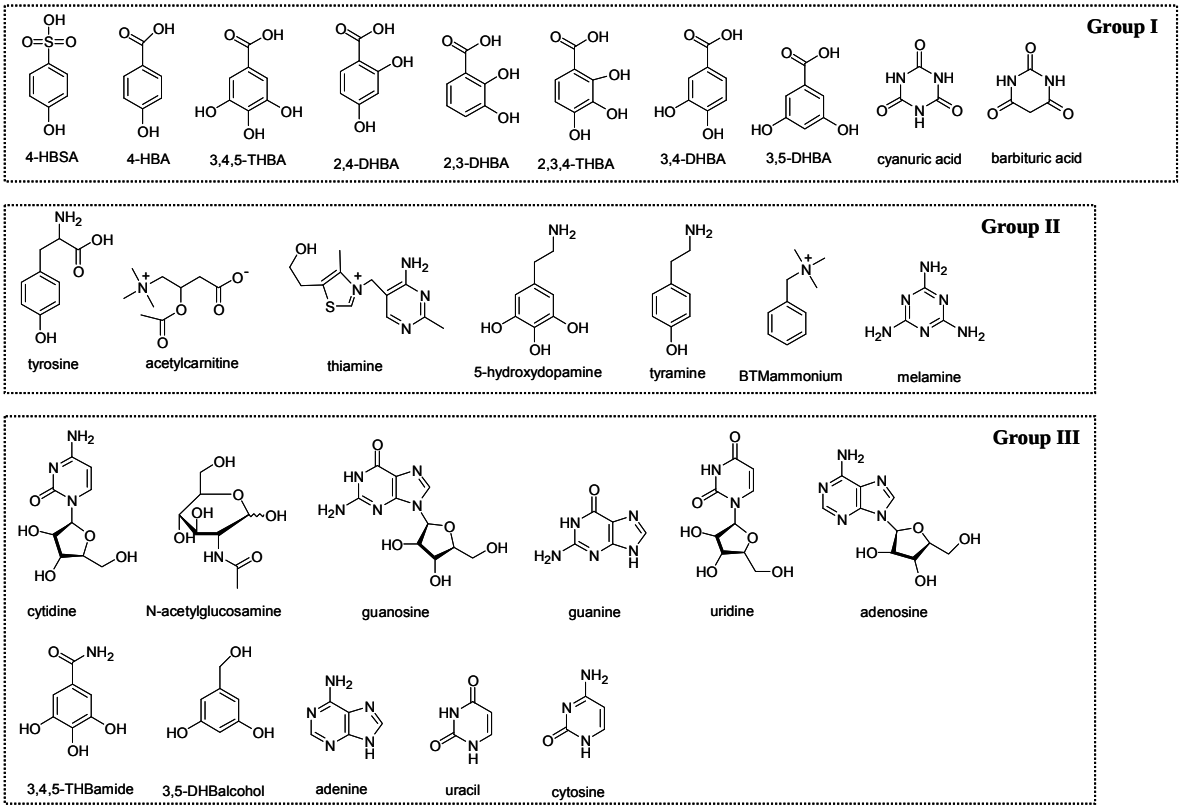


Figure 2 The structural formulas of test compounds.



(Abbreviation HBSA: hydroxybenzenesulfonic acid, HBA: hydroxybenzoic acid, DHBA: dihydroxybenzoic acid, THBA: trihydroxybenzoic acid)

Figure 3 orthogonal plotting of retention factors (ln *k vs* ln *k*) obtained on bare silica and 3.67 µmol.m^{-2}. Mobile phase: ACN/100mM NH4Ac in H2O pH 6 (90/10, v/v)

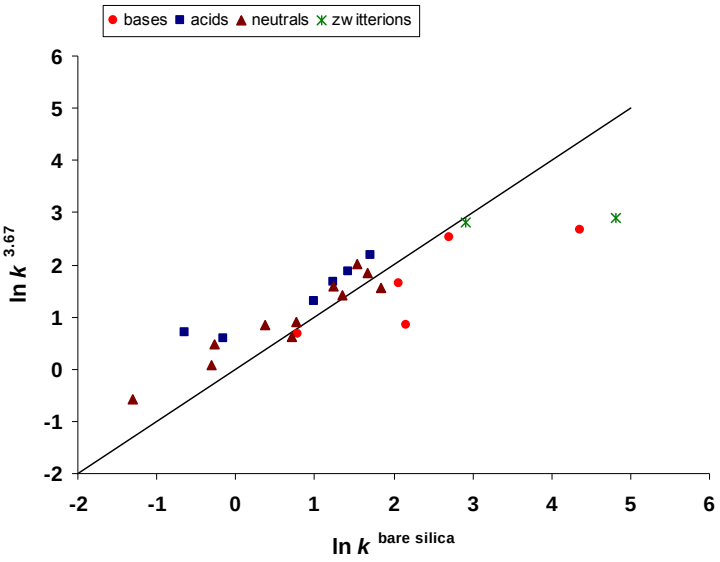
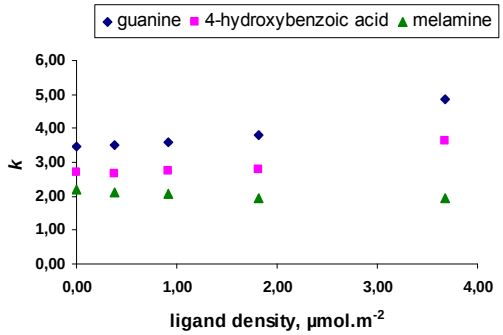


Figure 4

(4a) Retention factor *k* as a function of ligand density. Mobile phase: ACN/100mM NH4Ac in H2O pH 6 (90/10, v/v).



(b) Illustration of reversals of elution order resulting from an increase of ligand density.

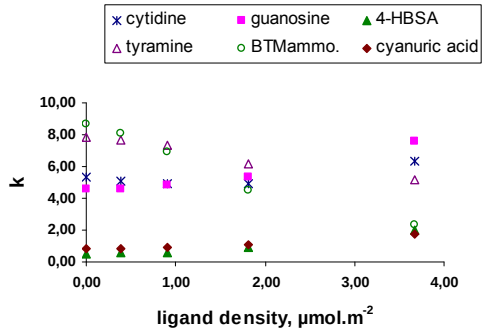
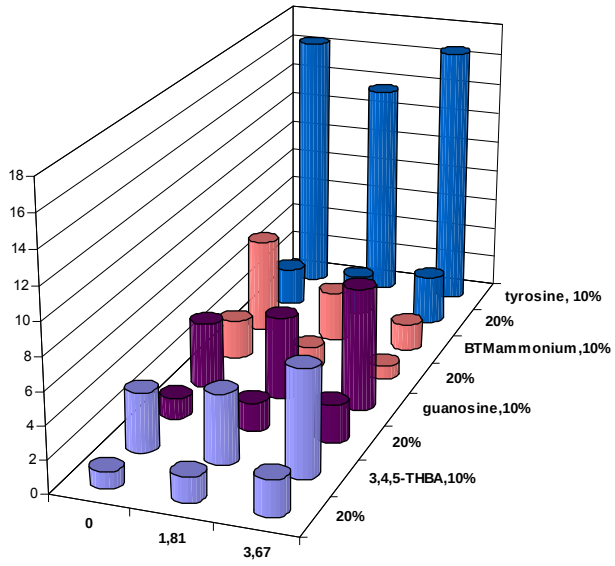


Figure 5

(5a) Illustration of water content in eluent on retention of acidic, basic, neutral and zwitterionic solutes. Mobile phase: ACN/H2O (90/10 or 80/20, v/v) with 10mM ammonium formate pH 7 before mixing



(5b) Illustration of response factor of retention for acidic, basic, neutral and zwitterionic solutes to the variation of water fraction in eluent from 10 to 20%.

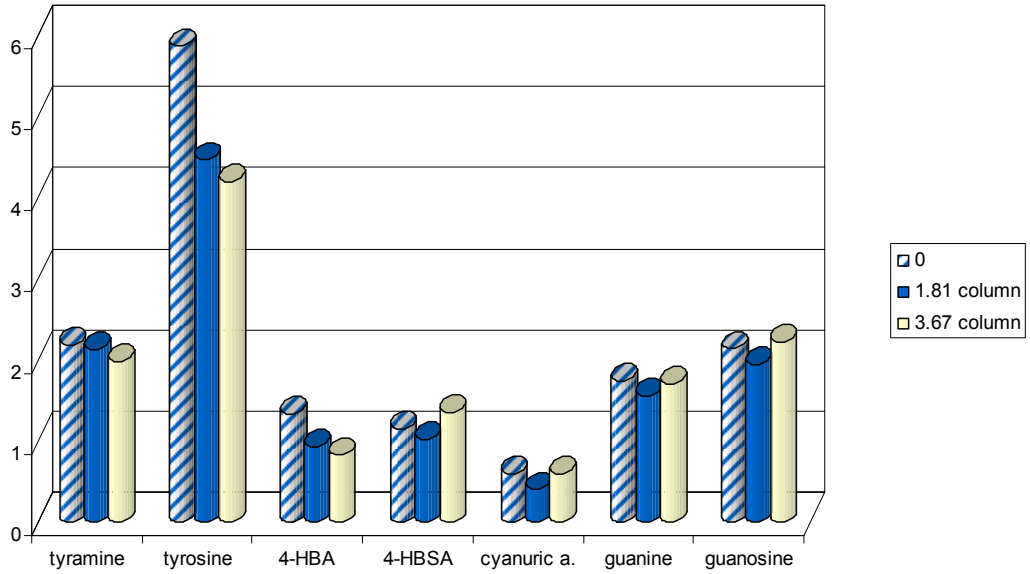
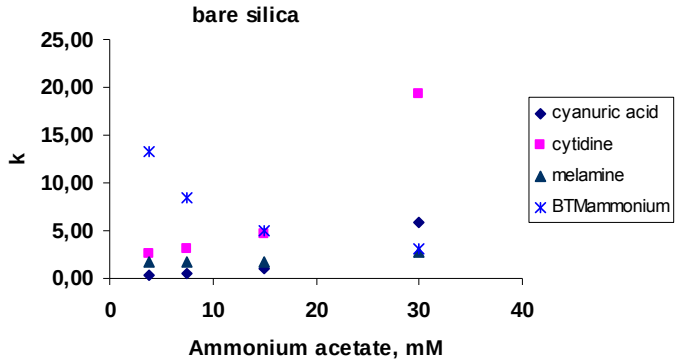


Table 1 retention factors of different benzoic acid analogue on three columns. Mobile phase: ACN/100mM ammonium formate pH 7 (90/10)

surface coverage	2,3-DHBA	2,4-DHBA	2,3,4-THBA	4-HBA	3,4-DHBA	3,5-DHBA	3,4,5-DHBA
µmol.m^{-2}	k	k	k	k	k	k	k
0	0,61	0,86	1,22	1,68	2,34	2,99	3,67
1,81	0,9	1,21	1,83	1,78	2,62	3,37	4,34
3,67	1,44	1,85	3,06	2,16	3,44	4,87	6,57

Figure 6

(6a) The effect of ammonium acetate concentration on retention factor on bare silica phase.



(6b) The effect of ammonium acetate concentration on retention factor on propylurea-bonded silica phase with ligand density 3.67 µmol.m^{-2}.

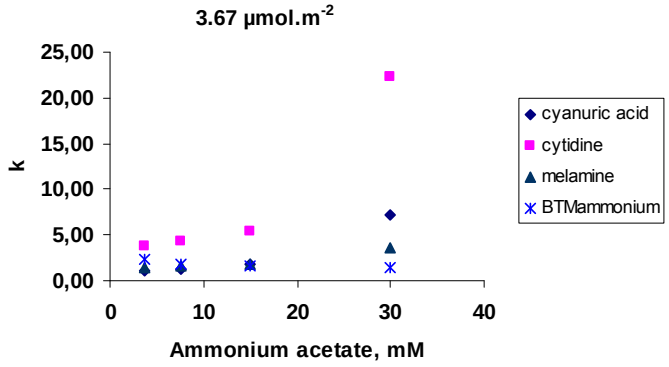
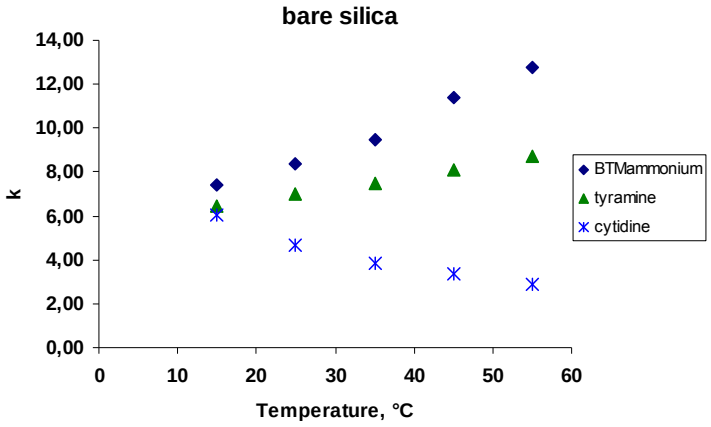
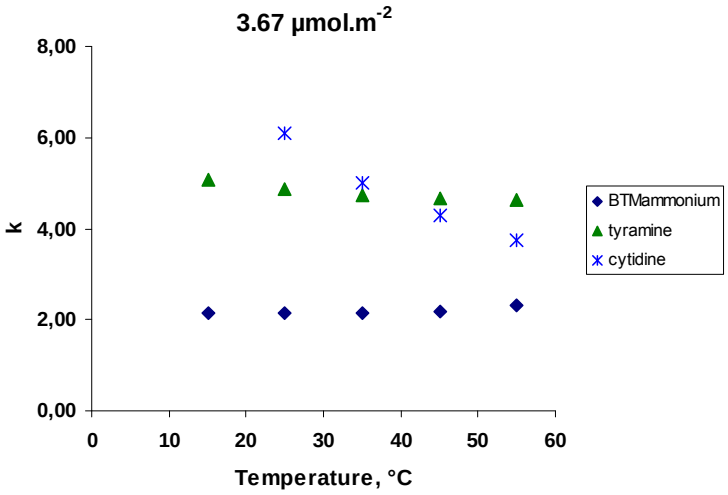


Figure 7

(7a) The effect of column temperature on retention factor on bare silica phase.



(7b) The effect of column temperature on retention factor on propylurea-bonded silica phase with ligand density 3.67 µmol.m^{-2}.



ADDENDUM #6

(Summary of developed materials)

Development of materials

1 Optimisation of the sulphur immobilisation based on trivinyl-silica

1. *Preparation of trivinyl silica* with trivinylchlorosilane in toluene (silica:silane=(4:1), imidazol 1.3eq. to silane), **Batch: WU060307**
2. *Endcapping of trivinyl silica* with trimethylsilylimidazol in toluene (silica:imidazol=3:1), **Batch: WU080307_1, *Start material*: material 1**

The following modifications are based on ec. trivinyl silica (WU080307_1)

A: sulphur-immobilisation with 1,3-propandithiol (PDT)

3. Cross-linking in MeOH with 20-mol% AIBN regarding to vinyl-groups
   Batch: WU080507_1, *Start material*: material 2
4. Modification with **PDT** in MeOH, (-SH:vinyl=**5:1,** AIBN **5-mol%** to vinyl),
   Batch: WU080507_2, *Start material*: material 3
5. Reduction of PDT silica with 5 mM tricarbonic acid phosphine
   (40 mg silica with 8 mL 5mM phosphine for 10min at room temperature)
   Batch: WU100507, *Start material*: material 4
6. Modification with **PDT** in MeOH with **acetic acid (2 mmol/g silica)**
   (-SH:vinyl=**5:1,** AIBN **5-mol%** to vinyl), **Batch: WU230507_1,**
   ***Start material*: material 3**

B: optimisation of sulphur-immobilisation with D,L-dithiothreitol (DTT)

7. Modification with **DTT** in MeOH with **10-mol%** AIBN regarding to vinyl
   (-SH:vinyl=**0.6:1**), **Batch: WU110407, *Start material*: material 2**

7*. Modification with **PDT** in MeOH with AIBN **5-mol%** to vinyl
   (-SH:vinyl=**6:1,**), **Batch: WU310709_2,**
   ***Start material*: material 7**

8. Modification with **DTT** in MeOH with **10-mol%** AIBN regarding to vinyl
   (-SH:vinyl=**1.2:1**), **Batch: WU170407, *Start material*: material 2**
9. Modification with **DTT** in MeOH with **10-mol%** AIBN regarding to vinyl
   (-SH:vinyl=**2.4:1**), **Batch: WU230407_3, *Start material*: material 2**

C: Coating of D,L-dithiothreitol (DTT) onto trivinyl silica (WU170807)

M_0. trivinyl silica. It was at first with 20-mol% AIBN **cross-linked** and then with hexamethyldisilazan **endcapped**. **Batch: WU170807**

10. **800 µmol DTT/g silica** in MeOH onto trivinyl slica coated and in toluene suspended, with **10-mol% AIBN** regarding to vinyl-groups at 80°C for 6h **Batch: WU111007, *Start material*: material M_0**
11. **1,6 mmol DTT/g silica** in MeOH onto trivinyl slica coated and in toluene suspended, with **20-mol% AIBN** regarding to vinyl-groups at 80°C for 6h **Batch: WU221007, *Start material*: material M_0**

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
1	trivinyl silica	WU060307	7,92	1,1	0,22			
2	endcapping	WU080307_1	8,32	1,22	0,09		3300	
3	cross-linking	WU080507_1	8,53	1,33	0,08			
4	PDT treatment	WU080507_2	10,64	1,87	0,08	4,9	764	420
5	reduction	WU100507						429
6	PDT treatment	WU230507_1	10,98	1,94	0,07	6,04	942	478
7	DTT treatment	WU110407	10,38	1,76	0,07	2,5	391	100
7*	PDT treatment	WU310709_2	9,54	1,65	0,06	2,91	64	121
8	DTT treatment	WU170407	10,04	1,7	0,05	2,84	443	122
9	DTT treatment	WU230407_3	10,68	1,67	0,07	4	624	195
M_0	trivinyl silica	WU170807	8,15	1,19	0,14			
10	DTT coating	WU111007	4,28	1,54	0,13	2,52	393	130
11	DTT coating	WU221007	10,29	1,82	0,14	3,77	588	235

2 Optimisation of AQ/AT-RPWAX and t-BuCQN immobilisation

12. PDT immobilisation of ec. cross-linked trivinyl silica in MeOH with **5-mol%** AIBN to vinyl and PDT (-SH:vinyl= **6:1**), **Batch: WU020507_1**

The following modifications are based on ec. cross-linked trivinyl silica (WU020507_1, 567 µmol/g)

13. Immobilisation of AQ-RPWAX ligand (WB08-08-2005) in MeOH ligand **625 µmol/g silica** and **AIBN 10-mol%** regarding to ligand **Batch: WU070507_1, *Start material*: material 12**
14. Immobilisation of AT-RPWAX. HCl ligand (WU020507_2) in MeOH ligand **2 mmol/g silica**, **AIBN 20-mol%** regarding to ligand and **acetic acid 2 mmol/g silica**, **Batch: WU090507, *Start material*: material 12**

M_1: PDT-silica, (-SH:vinyl= **5:1**), **Batch: WU140607**

15. Immobilisation of AT-RPWAX. HCl ligand (WU230507_2) in 2-propanol/water (30/70), PDT silica (**524 µmol/g**), ligand 650 µmol/g silica, **20-mol% V-50 Starter** regarding to ligand and acetic acid 2 mmol/g silica, **Batch: WU180607, *Start material*: material M_1**
16. Immobilisation of t-BuCQN selector in MeOH
    Selector **625 µmol/g silica**, **AIBN 10-mol%** regarding to ligand, **Batch: WU070507_2, *Start material*: material 12**
17. Immobilisation of t-BuCQN selector in MeOH
    Selector **600 µmol/g silica**, **AIBN 10-mol%** regarding to ligand, **acetic acid 2 mmol/g silica**, **Batch: WU300507_1, *Start material*: material 6**

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
12	PDT treatment	WU020507_1	10,92	1,97	0,07	6,2	967	567
13	AQ-RPWAX	WU070707_1	17,12	2,83	1	5,28	357	61
14	AT-RPWAX	WU090507	18,28	2,78	1,17	5,05	418	64
M_1	PDT silica	WU140607	9,84	1,7	0,09	5,28	823	524
15	AT-RPWAX	WU180607	17,42	2,87	1,1	4,67	390	96,4
16	t-BuCQN	WU070507_2	19,37	2,79	1,47	5,04	350	105
17	t-BuCQN	WU300507_1	18,04	2,59	1,26	5,23	300	127

3 Development of cysteamine-modified packing based on trivinyl silica

18. trivinyl silica (WU080307_1, **endcapped**, 3.3 mmolvinyl/g), cysteamine. HCl (2 mmol/g) in MeOH with acetic acid (2 mmol/g) and **AIBN 3-mol%** regarding to vinyl-groups, 16h.
    Batch: WU270307, *Start material*: material 2
19. WU270307 (one time modified), cysteamine.HCl (2 mmol/g) in MeOH with acetic acid (2 mmol/g) and **AIBN 20-mol%** regarding to vinyl-groups, 20h. **Batch: WU030407, *Start material*: material 18**
20. Stresstest 1 to cysteamine-silica (WU030407), 100 mg silica, 100 mg urea, 1 mL 7M ammonia solution (pH 12.7), at **60°C**, shaking for **1h**, **Batch: WU290607_1, *Start material*: material 19**
21. Stresstest 2 to cysteamine-silica (WU030407), 100 mg silica, 100 mg

urea, 1 mL 7M ammonia solution (pH 12.7), at **room temperature**, shaking for **8h**, **Batch: WU290607_2, *Start material*: material 18**

M_2: PDT-silica, cross-linking of trivinyl silica with 200 µmol/g PDT, **Batch: WU220807_2, *Start material*: material M_0**

22. trivinyl silica (3.3 mmolvinyl/g, WU220807_2, **ec.** TV-silica **cross-linked** with PDT 200 µmol/g silica), cysteamine.HCl (2 mmol/g) in MeOH with acetic acid (2 mmol/g) and AIBN 20-mol% regarding to vinyl-groups, **6h Batch: WU240807, *Start material*: material M_2**
23. WU240807 (one time modified), cysteamine.HCl (2 mmol/g) in MeOH with acetic acid (2 mmol/g) and **AIBN 20-mol%** regarding to vinyl-groups, **20h. Batch: WU240807b, *Start material*: material 22**
24. Modification of epoxydecane onto cysteamine-silica in ACN. Cysteamine silica (WU240807b), triethylamine (1.2 mmol/g silica), 1,2-epoxydecane (2 mmol/g silica), at 80°C for 20h, **Batch: WU110907, *Start material*: material 23**

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
18	cysteamine	WU270307	8,3	1,39	0,09	0,17	54	
19	cysteamine	WU030407	8,88	1,68	0,71	1,57	491	
20	stresstest 1	WU290607_1	16,55	3,13	1,47	3,23	???	
21	stresstest 2	WU290607_2	7,76	1,42	0,69	1,48	462	
M_2	cross-linking	WU220807_2	8,41	1,31	0,14	0,84	131	0
22	cysteamine	WU240807	7,91	1,24	0,29	1,13	107	
23	cysteamine	WU240807b	9,06	1,53	0,79	1,74	460	
24	23+epoxydecane	WU110907	10,15	1,68	0,58	1,7	90	

4 Development of arginine-modified silica

25. Modification of 2-mercaptoethanol (MP) onto trivinyl silica (WU080307_1, with trimethylsilylimidazol endcapped) in MeOH. 2-mercaptoethanol (10-mol eq. to vinyl-groups), 4-mol% AIBN regarding to vinyl-groups, at 65°C for 16h. **Batch: WU200307, *Start material*: material 2**
26. Modification of p-toluenesulfonyl chloride onto MP-silica in **dichloro-methane**. Toluenesulfonyl chloride (1.5-mol eq. to MP-groups), N-

ethyldiisopropylamine (1.5-mol eq. to MP-groups), at **40°C** for **6h**.

Batch: WU220307, *Start material*: material 25

27. Modification of p-toluenesulfonyl chloride onto WU220307 in **pyridine**. Toluenesulfonyl chloride (1.5-mol eq. to MP-groups), at **85°C** for **20h**.

Batch: WU020407_2, *Start material*: material 26

28. Modification of methylsulfonyl chloride onto MP-silica in dichloro-methane. Methylsulfonyl chloride (1.5-mol eq. to MP-groups), N-ethyldiisopropylamine (1.5-mol eq. to MP-groups), at 40°C for 6h.

Batch: WU260307, *Start material*: material 25

29. Modification of 4-nitrobenzylchloroformate onto MP-silica in **pyridine**. 4-nitrobenzylchloroformate (1.5-mol eq. to MP-groups), at 85°C for 20h.

Batch: WU100407, *Start material*: material 28

30. Modification of hydrazine onto 4-nitrobenzylchloroformate silica in **ACN**. Hydrazine (11-mol eq. to chloroformate), at room temperature for 6h.

Batch: WU230407_2, material 100 mg, *Start material*: material 29

31. Modification of **L-arginine base** onto **4-nitrobenzylchloroformate silica** in **dimethylsulfoxide**. L-arginine (11-mol eq. to chloroformate), at room temperature for 6h.

Batch: WU150507_1, *Start material*: material 29

32. Modification of **L-arginine.HCl** onto 4-nitrobenzylchloroformate silica in **dimethylsulfoxide**. L-arginine.HCl (11-mol eq. to chloroformate), **triethylamine** (5-mol eq. to arginine)at room temperature for 6h.

Batch: WU150507_2, *Start material*: material 29

33. Modification of **L-arginine. 2HCl** onto 4-nitrobenzylchloroformate silica in **dimethylsulfoxide**. L-arginine.2HCl (11-mol eq. to chloroformate), triethylamine (5-mol eq. to arginine), at room temperature for 6h.

Batch: WU150507_3, *Start material*: material 29

M_3. PDT-silica, (-SH:vinyl= **5:1**), **Batch: WU160507**

34. Modification of **allylamine** onto PDT-modified silica in isopropanol/water (30/70) with 20-mol% regarding to allylamine V-50 Starter, at 70°C for 6h. **Batch: WU270607, *Start material*: material M_3**

35. Modification of **L-arginine base** onto **allylamine-silica** in

dimethylsulfoxide. L-arginine base (11-mol eq. to chloroformate), triethylamine (5-mol eq. to arginine), at room temperature for 6h. **Batch: WU280607_1, *Start material*: material 34**

36. Modification of **L-arginine.HCl** onto **allylamine-silica** in **dimethylsulfoxide**. L-arginine.HCl (11-mol eq. to chloroformate), triethylamine (5-mol eq. to arginine), at room temperature for 6h. **Batch: WU280607_2, *Start material*: material 34**
37. Modification of **L-arginine.2HCl** onto **allylamine-silica** in **dimethylsulfoxide**. L-arginine.2HCl (11-mol eq. to chloroformate), triethylamine (5-mol eq. to arginine), at room temperature for 6h. **Batch: WU280607_3, *Start material*: material 34**

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
25	mercaptoethanol	WU200307	9,67	1,79	0,033	2,52	786	
26	toluenesulfonyl	WU220307	9,7	1,76	0,037	2,53	3	
27	toluenesulfonyl	WU020407_2	14,03	2	0,761	3,15	???	
28	methylsulfonyl	WU260307	9,67	1,74	0,036	2,57	12	
29	nitrobenzylchloroformate	WU100407	10,55	1,68	0,524	2,2	348	
30	29 + hydrazine	WU230407_2	10,04	1,56	0,92	2,1	328	
31	29 + L-arginine base	WU150507_1	9,96	1,54	0,537	2,13	0	
32	29 + L-arginine.HCl	WU150507_2	10,49	1,62	0,564	2,23	0	
33	29 + L-arginine.2HCl	WU150507_3	9,85	1,52	0,51	2,08	0	
M_3	PDT treatment	WU160507	9,75	1,66	0,094	5	780	460
34	allylamine	WU270607	10,86	1,9	0,217	5,55	155	
35	34 + L-arginine base	WU280607_1	11,02	1,91	0,292	5,24	13	
36	34 + L-arginine.HCl	WU280607_2	10,93	1,88	0,281	5,46	11	
37	34 + L-arginine.2HCl	WU280607_3	11,06	1,93	0,36	5,45	28	

5 Development of Isocyanate-modified silca

38. Modification of 2-mercaptoethanol onto trivinyl silica (WU080307_1, with trimethylsilylimidazol endcapped) in MeOH. 2-mercaptoethanol (10-mol eq. to vinyl-groups), 5-mol% AIBN regarding to vinyl-groups, at 65°C for 7h. **Batch: WU250407, *Start material*: material 2**
39. Immobilisation of phenylisocyanate onto mercaptoethanol-silica in toluene. Phenylisocyanate (2 mmol/g silica), at 60°C for 4h. **Batch: WU260407, *Start material*: material 38**

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
38	mercaptoethanol	WU250407	9,76	1,72	0,024	2,66	829	
39	isocyanate	WU260407	12,08	1,83	0,508	2,52	363	

6 Development of sulfonic acid-type silica

The development was based on 1,3-propandithiol-modified silica (PF020707_1)

M_4. PDT-silica, **Batch: PF020707_1**

40. Oxidation of PDT-immobilized silica with **H_2O_2** in MeOH/H_2O (4:1). H_2O_2 (1 g/g silica), at room temperature for 5h.

    Batch: WU040707, *Start material*: material M_4

41. Oxidation of WU040707 with **performic acid** in MeOH with formic acid (20 mmol/g silica). Performic acid (formic acid: H_2O_2= 5:1, mol:mol, at room temperature for 2h, 80-mol eq. regarding to –SH-groups), at room temperature for 5h.

    Batch: WU300807, Column, *Start material*: material 4

42. Oxidation of PDT with **performic acid** in MeOH with formic acid (20 mmol/g silica). Performic acid (80-mol eq. regarding to –SH-groups), at room temperature for 5h.

    Batch: WU211107, Material: 3 g, *Start material*: material M_4

43. **Coating** of 3-mercaptopropansulfonate onto trivinyl-silica in **MeOH**. 3-mercaptopropansulfonate (1.6 mmol/g silica), AIBN 10-mol% regarding to vinyl-groups, in **toluene** at 80°C for 6h.

    Batch: WU141107, *Start material*: material M_0

44. Modification of 3-mercaptopropansulfonate onto trivinyl-silica in **isopropanol/H_2O (30/70)** with **V-50 Starter**. 3-mercapto-propansulfonate (1.6 mmol/g silica), **acetic acid** (2 mmol/g silica), at 70°C for 6h. **Batch: WU161107, *Start material*: material M_0**

44*: Modification of 3-mercaptoacetic acid onto trivinyl-silica in MeOH with AIBN 10-mol% regarding to vinyl-groups,. 3-mercaptoacetic acid (3 mmol/g silica), at 65°C for 6h.

Batch: WU300709, *Start material*: material M_0

Analytical data

							µmol/g	µmol/g	**Micro-titration**
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**	µmol/g
M_4	propandithiol	PF020707_1	10,25	1,8	0,077	4,76	742	403	
M_0	trivinyl silica	WU170807	8,15	1,19	0,144				
40	oxidation w. H_2O_2	WU040707	9,91	1,58	0,092	5,195			30
41	40 + performic acid	WU300807	9,3	1,75	0,102	4,56		88	255
42	PF020707_1+ performic acid	WU211107	8,97	1,61	0,096	4,03		83	360
43	Mercaptopropan-sulfonate	WU141107	8,45	1,33	0,173	0,334	52		
44	Mercaptopropan-sulfonate	WU161107	9,74	1,67	0,523	3,3	515	??? N	little
44	Mercaptopropan-sulfonate	WU161107	9,74	1,67	0,523	3,3	515	??? N	little
44*	mercaptoactic acid	WU300709	8,95	1,41	0,098	2,05	640		

7 Development of azide-modified silica and diol-type polar phases

45. Preparation of glycidoxypropyl-silica. 3-glycidoxypropyl-trimethoxysilane (2 mmol/g silica), 4-dimethylaminopyridine (1.5-mol% regarding to silane), in toluene at 135°C for 20h. **Batch: WU110308**
46. Immobilisation of azide onto the glycidoxypropyl-silica in MeOH. Sodium azide (5 mmol/g silica), at **room temperature** for **4 days**.

    Batch: WU200308, *Start material*: material 45
47. Immobilisation of azide onto the glycidoxypropyl-silica in MeOH. Sodium azide (5 mmol/g silica), at **50°C** for **18h**.

    Batch: WU290708, *Start material*: material 45
48. Preparation of glycidoxypropyl-azide-silica n MeOH. Daisogel 120-5 µm, 3- glycidoxypropyl-trimethoxysilane (silica:silane=3:1), Sodium azide (2.5 mmol/g silica), at **60°C** for **16h**.

    Batch: WU080709, *Start material*: material Daisogel SP-120-5µ
49. Immobilisation of thioglycerol onto the glycidoxypropyl-silica in MeOH/H_2O (50/50). 1-thioglycerol (1.5 mmol/g silica), triethylamine (1 mmol/g silica), at 60°C for 20h.

    Batch: WU090408, *Start material*: material 45

50. Oxidation of thioglycerol silica (WU090408) with **H_2O_2** in H_2O. H_2O_2 (1 g/g silica), at room temperature for 5h.

Batch: WU170408, *Start material*: material 49

50*. Preparation of azide-modified silica. 3g Daisogel (5µm), 3-glycidoxypropyltrimethoxysilane (1.5 mmol/g silica), 500mg Sodium azide (2.5 mmol/g silica), in MeOH at room temperature for 4 days.

Batch: WU310709_1, *Start material*: Daisogel (5µm)

Analytical data

							µmol/g	µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**	**DPDS**
45	glycidoxypropyl	WU110308	8,09	1,42			842	
46	45 + azide, room temp.	WU200308	6,99	1,25	1,63		380	
47	45 + azide, 50°C	WU290708	6,9	1,36	1,11		264	
48	daisogel+silane+azide	WU080709	4,61	1,15	1,003		239	
49	45 + thioglycerol	WU090408	8,23	1,63	0,111	1,52	475??	
50	49 + H2O2	WU170408	7,82	1,56	0,1	1,44		
50*	daisogel+silane+azide	WU310709_1	5,19	1,09	0,753		180	

8 Development of trivinyl-based silica for immobilisation of t-BuCQN selector

The development was based on trivinyl-silica (**WU170807**, cross-linked with 20-mol% AIBN at first and then endcapped, **3.3 mmol vinyl/g silica**).

51. Immobilisation of methyl thioglycolate in MeOH. methyl thioglycolate (3 mmol/g silica), AIBN 10-mol% regarding to vinyl-groups, at 60°C for 6h.

Batch: WU030408, *Start material*: material M_0

52. Immobilisation of diallylamine onto thioglycolate-silica (WU030408) in toluene. Diallylamine (2-mol eq. to thioglycolate), at 60°C for 24h.

Batch: WU140408, *Start material*: material 51

M_5. Immobilisation of methyl thioglycolate in MeOH. methyl thioglycolate (3 mmol/g silica), AIBN 10-mol% regarding to vinyl-groups, at 60°C for 20h. **Batch: WU150408, *Start material*: material M_0**

53. Immobilisation of tetraethylenepentamine (TEPA) onto thioglycolate-silica (WU150408, 9.62% C, 0.098% N, 2.26% S, 705 µmol/g) in

toluene. TEPA (400 µmol/g silica), at 130°C for 20h.

Batch: WU290408, *Start material*: material M_5

Analytical data

							µmol/g
material	chemistry	Batch Nr.	C%	H%	N%	S%	**CHNS**
51	methyl thiolycolate	WU030408	9,51	1,49	0,116	2,07	645
52	51 + diallylamine	WU140408	9,56	1,48	0,098	2,08	0
M_5	methyl thiolycolate	WU150408	9,62	1,53	0,098	2,26	705
53	thioglycolate silica + TEPA	WU290408	10,47	1,74	1,235	2,1	176

Summary

Over the past years, Hydrophilic Interaction Chromatography (HILIC) faced gaining popularity for the analysis of a wide variety of polar compounds due to the growing demand of analysis of highly polar compounds in *life science* fields, such as pharmaceutical sciences, bioanalytics, biomedical. The main objective of this thesis was to develop and evaluate polar-modified silica-based stationary phase for applications in the HILIC mode and to gain an insight into the HILIC retention mechanism which has been barely explored yet and seems to be quite complex.

In a first attempt, four stationary phases having non-charged ligands with hydroxyl as well as sulfide- or sulfoxide-groups bonded to silica were prepared. The synthesis concept of these packings was based on an immobilisation of 2-mercaptoethanol (ME phase) and 1-thioglycerol (TG phase), respectively, onto vinylised silica by radical addition. Subsequent on-phase oxidation of the embedded sulphide groups with hydrogen peroxide delivered sulfoxide-groups (MEO and TGO phases). The characterisation was based on the effect of parameter of stationary phase *i.e.* type of polar functionalisation, surface coverage of ligand and silanol background respectively and the parameter of mobile phase *i.e.* type and amount of protic modifier, type and amount of salt, pH value and column temperature.

These new developed diol-type stationary phases offered HILIC chromatographic selectivity towards the separation of nucleosides, nucleobases and water soluble vitamins. Compared to commercial diol-type HILIC packings complementary selectivity was found which may be an indication for different retention mechanisms being at work. These home-made more polar phases generally exhibited a stronger retention of polar compounds while differences in selectivity were observed depending on the type of polar ligand functionality (hydroxyl *vs.* sulfoxide). Thus, distinct stationary phase effects of the home-made polar packings were noticed in the HILIC mode. In addition, the sulfoxide functionality in ligand was found to be very useful to create new retention and selectivity in HILIC separation. Furthermore, despite the presence of polar functionalities in the C_4 and C_5 chains the developed diol-type phases were also conceptually useable in the reversed-phase (RP) elution mode with respect to CH_2-increment selectivity (offering a multimodal separation capability).

When the chromatographic separation is carried out on a silica-based stationary phase, residual silanol activity is one unavoidable parameter to be concerned. Silanol activity is a well known and an already detailed explored parameter in RP chromatography (effect on peak shape) while there is almost no information available how silanol interactions contribute to

retention and selectivity characteristics of polar bonded phases operated in the HILIC elution mode. For this reason, a systematic study was designed in the course of this thesis. A set of acidic, basic, zwitterionic as well as non-charged compounds were run with different mobile phases on a bare silica column as well as on propylurea-bonded silica packings having a surface coverage between 0 and 3.67 µmol.m^{-2}.

Pronounced effects of surface coverage of bonded silica phase on retention and selectivity were observed in the HILIC mode. For example, when the ligand density was increased from 0 to 3.67 µmol.m^{-2}, retention gradually increased for acidic and non-charged solutes while it decreased for zwitterionic and in particular for basic solutes. The gain in retention upon increasing surface coverage was in line with a result of partitioning retention process while the loss in retention may possibly own to the attendance of strong attractive silanol-solute interactions, even as main retention mechanism. A stronger susceptibility to an increasing ligand density was found for acidic solutes than that for non-charged analytes which can be explained by a reduction of strong repulsive silanol-solute interactions on densely bonded phase. Accordingly, silanolphilic interactions are involved in the HILIC retention process, especially for charged and chargeable solutes.

With regard to effects of mobil phase parameters, further study was investigated to explore the role of the type of protic modifier in the HILIC separation process in which the water fraction in acetonitrile-rich eluents was exchanged by various alcohols (1,2-ethanediol, methanol, ethanol). On a set of non-charged bonded phase (most of them being of diol-type) as well on bare silica these non-aqueous but polar elution conditions resulted in a stronger retention of hydrophilic compounds compared to more common aqueous eluents. Thereby, the polarity of the protic modifier adopted a primary role in determining the extent of retention and, generally, increased retention was found in the order water < 1,2-ethanediol < methanol < ethanol. Besides effects on retention, chromatographic selectivity was also found to be significantly affected by the type of alcohol modifier. For example, under isoelutropic conditions compared to an aqueous HILIC eluent, mobile phase with methanol as protic modifier caused an inverted elution order of guanosine and guanine. This indicated the occurrence of solvent-specific selectivity effects in HILIC which is in accordance with partition like mechanism.

The amount of protic modifier in mobile phase had also significant effects on retention and selectivity. Upon decreasing the amount of protic modifier in eluent (< 40%, v/v) the retention was increased for all solutes in both AQ-HILIC and NA-HILIC elution mode. The extent of influence for individual solute was not in a uniform trend resulting in a complex effect on

selectivity.

Except the effects of the type and amount of protic modifier, the effects of other parameters of mobile phase, namely pH value of buffer, type and amount of salt and column temperature were also characterised in HILIC. The pH value of buffer (in the range of pH 3.0-8.0) did not cause significant retention and/or selectivity shifts for non-charged polar solutes on non-charged bonded phases and the retention varied less than 10% while for charged und chargeable solutes the change of retention was more than 10%. Different pH values of buffer led to different eluent ${}^{s}_{w}$pH values (measured pH value after addition of organic solvent) which can cause an effect on the ionisation states of residual silanols and solutes, especially for charged and chargeable compounds, resulting in a change in retention and selectivity.

When the salt concentration of ammonium acetate was increased from 3.75 mM to 30 mM (90% ACN) in the mobile phase, retention for all acidic, weak basic and non-charged solutes was increased in a gradual manner on non-charged bonded phases under aqueous HILIC elution conditions while a decrease in retention was found for strong bases. Under non-aqueous HILIC elution conditions, both increase and decrease in retention for non-charged solutes were observed. An increase in retention upon increasing salt concentration is consistent with the result from a retention mechanism primarily driven by 'hydrophilic interactions while a loss in retention is to be expected for a mechanism driven by attractive 'ionic interactions'. Furthermore, the different nature of salts caused different eluent ${}^{s}_{w}$pH values which could induce different chromatographic effects on retention and selectivity.

The temperature of the column and separation system, respectively, is of course another effective parameter on retention and selectivity. With increasing column temperature, most acidic and non-charged solutes showed a decrease in retention under aqueous HILIC elution conditions while an increase in retention was observed for strong bases and zwitterionic compounds. This was strongly towards different retention mechanisms for different solutes. In non-aqueous HILIC elution mode, regardless of the type of protic modifier, a decreased retention was found for all investigated non-charged compounds at higher temperature while the extent was distinctly different with different protic modifier (differential thermodynamic compensation effect of protic modifiers).

Beside the study of chromatographic characteristics and retention mechanisms of non-charged silica-bonded polar packings, another part of the present thesis were the chromatographic characterisations of RP/WAX columns in HILIC and RP elution modes and the study of involved HILIC retention process on such charged columns. Earlier a set of RP/WAX phases with different RP/WAX ligand, namely (i) N-undecanoyl 3-aminoquinuclidine; (ii) N-

undecanoyl 3-aminotropane, (iii) N,N-dimethyl-N-10-undecanoyl-1,2-ethanediamine and (IV) N-butanoyl-(2S, 4S, 5R)-2-aminomethyl-5-[(2-octylthio) ethyl]-quinuclidine were developed in our own group and in this study they were comparatively evaluated with commercial columns (WAX-type, RP-type, RP/WAX-type, HILIC-type). In RP elution mode, all these developed RP/WAX columns exhibited sufficient hydrophobicity with $k^{\text{pentylbenzene}}$ = 6.4~18.4 and anion-exchange capability with respect to the ability to retain polar acid *o,o*-diethylthiophosphate(DETP) k^{DETP} = 12.69~30.75. But, the DETP was eluted at the void time on the single-mode Synergi Fusion RP column. On WAX-type phases such as Luna amino DETP could be well retained k^{DETP} = 4.39 while there was no CH_2-increment selectivity observed. The RP/WAX materials provide unique retention and selectivity profiles in RP elution mode compared to the single-mode stationary phase (RP phase and WAX phase).

Additionally, these RP/WAX columns showed also good HILIC separations capabilities in term of xanthins and nucleosides, while some critical peak pairs and also deteriorated peak shapes were observed on typical HILIC columns such as TSK Gel Amide-80, ZIC-HILIC and Polysulfoethyl A. The multimodal separation properties (hydrophobic and ionic) of RP/WAX phases offered complementary HILIC separation capability. With regard to the retention mechanism, electrostatic interactions were superimposed on the hydrophilic interactions composed of adsorptive and partitioning interactions.

Besides extending knowledge on the phase evaluation for HILIC applications and the mechanisms involved in the HILIC retention process, a further part of the PhD thesis was devoted to a practical application of a home-made as well as of a set of commercial polar packings. *G. lucidum* is a medicinal fungus in Traditional Chinese Medicine (TCM) and it is well known owing to his valuable medical and therapeutic functions. Due to the wide range of *Ganoderma* species and the broad geographic origins, it is necessary to establish a suitable methodolody for discrimination, standardization and quality control of *G. lucidum*. The Chromatographic fingerprint analysis of *G. lucidum* was carried out in HILIC mode (TGO column, Luna HILIC, Polysulfoethyl A, TSK Gel Amide-80 and ZIC-HILIC) and RP mode (Zorbax SB-C_{18} column). The motivation of combining the HILIC and RP elution modes in this study was to extend the accessible polarity window of compounds and thus, to gain more discriminative information for *Ganoderma* sample identification.

TGO phase exhibited good ability for chemometric descrimination of some *Ganoderma* species (*G. lucidum, G. atrum, G. sinense, G. tsugae*) from *G. lucidum*. Different HILIC columns showed quite different peak patterns, while their usability for chemometric discrimination of *Ganoderma spp.* was largely similar. In other words, the nature of the polar

bonding only had a minor effect on their applicability for the present problem. In the HILIC elution mode, misclassifications of *Ganoderma spp.* were observed on all columns. For examples, two out of the five *G. sinense* samples were wrongly classified on TGO phase and four out of the five *G. sinense* could not be discriminated from *G. lucidum* on TSK Gek Amide-80 and Polysulfoethyl A columns. In the RP mode, wrong discriminations were found for *G. atrum* and *G. sinense* samples from *G. lucidum*. However, a combining of the data of HILIC mode and RP mode led to a 100% correct sample discrimination rate for the *Ganoderma spp.*. As it can be seen, multi-modal chromatographic fingerprint analysis (HILIC & RP) is more advantageous than a single-mode approach.

According to all results, a few findings can be outlined. Phase polarity and polar sites in ligand (hydroxyl, sulfoxide) were very useful to change HILIC characteristics. Silanophilic interaction was an influential factor in HILIC retention process, especially for charged and chargeable hydrophilic analytes. Besides the amount of protic modifier, the pH value of buffer, the nature and amount of salts and column temperature, the type of protic modifier was an effective tool in method development/optimisation for separation problems involving polar solutes. The HILIC retention mechanism was quite complex and it was supposed that the hydrophilic interactions comprised partitioning and weak adsorptive interactions such as hydrogen bonding, dipole-dipole interactions developed by direct solute-ligands interactions, solute-solvation shell on the surface or/and solute-silanols. For charged packings, electrostatic interactions were easily superimposed on the hydrophilic interactions.

Zusammenfassung

In den vergangenen Jahren hat die Hydrophilic Interaction Chromatography (HILIC) an Popularität für die Analyse einer Vielzahl von polaren Verbindungen aufgrund des wachsenden Bedarfs der Analyse der stark polaren Verbindungen in life-science Bereichen wie z.B. Pharmazie, Bioanalytik, Biomedizin gewonnen. Das Ziel der Doktorarbeit war die Entwicklung und die Evaluierung der polar modifiziert und Silica-basiert stationären Phasen für die Applikationen im HILIC-Modus. Durch eine systematische Herangehensweise versprachen wir uns ein besseres Verständnis der noch kaum untersuchten und offensichtlich komplexen HILIC Retentionsmechanismen.

In einem ersten Versuch wurden vier stationäre Phasen synthetisiert, deren Ligand Hydroxyl- sowie Sulfid- oder Sulfoxid-Gruppen enthielten. 2-Mercaptoethanol (ME-Phase) und 1-Thioglycerin (TG-Phase) wurden auf vinylisiertem Silica durch eine radikalische Additionsreaktion immobilisiert. Anschließend wurde eine on-phase Oxidation mit Wasserstoffperoxid durchgeführt, wobei Sulfid-Gruppen in Sulfoxid-Gruppen umgewandelt wurden (MEO und TGO Phasen). Der Einfluss der Parametern der stationären Phase (Typ der polaren Funktionalisierung, Ligandendichte, Silanol-Hintergrund) und der mobilen Phase (Art und Menge des protischen Modifiers, Art und Menge von Salzadditiven, pH-Wert, Temperatur) wurden analysiert.

Diese neu entwickelte diol-Typ stationäre Phasen zeigte eine sehr gut Eignung für die Trennungen von Nukleosiden, Nukleobasen und wasserlösliche Vitamine im HILIC Modus. Im Vergleich zu kommerziellen HILIC Säulen wurden komplementäre Selektivitäten gefunden, die auf Unterschiede im Retentionsmechanismus hindeuteten. Mehr polarer Phasen zeigten eine stärkere Retention von polaren Verbindungen, wobei eine unterschiedliche Selektivität in Abhängigkeit von der Art der polaren Funktionalität in Ligand (Hydroxyl *vs* Sulfoxide) beobachtete wurde. Daraus wird ersichtlich, dass die stationäre Phase einen signifikanten Effekt auf Retention im HILIC Modus hat. Außerdem wirkten die Sulfoxid Funktionalität in den Ligand sehr positiv auf die Generierung, was sich in Retention und Selektivität bemerkbar machte. Weiterhin waren trotz der Anwesenheit von polaren Funktionalitäten in den C_4 und C_5 Ligandenketten die entwickelte Diol-Typ Phasen auch im Reversed-Phase (RP) Modus mit Bezug auf eine CH_2-Inkrement Selektivität verwendbar(Multimodale Trennungsfähigkeit).

Wenn die chromatographische Trennung auf einer Silica-basiert stationäre Phase durchgeführt wird, ist die Silanol-Aktivität als wichtiger Parameter zu berücksichtigen. Silanol-Aktivität ist ein bekannter und bereits detailliert untersuchter Parameter im RP Modus (bezüglich des Peak-Shape). Aber es sind fast keine Information verfügbar wie die Silanol-Interaktionen die chromatographische Eigenschaften der polaren gebundenen stationären Phasen im HILIC Modus beeinflussen. Aus diesem Grund wurde eine systematische Untersuchung der Silanol-Aktivität im HILIC Modus entwickelt. Hierbei wurde eine Reihe von sauren, basischen, zwitterionischen sowie nicht-geladenen Verbindungen mit verschiedenen mobilen Phasen auf einer nackte Silica Phase sowie auf Harnstoff-gebundenen Phasen mit einer Ligandendichte zwischen 0 und 3.67 $\mu mol.m^{-2}$ aufgebracht. Ausgeprägte Effekte der Ligandendichte auf Retention und Selektivität wurden gefunden. Zum Beispiel war, wenn die Ligandendichte von 0 auf 3.67 $\mu mol.m^{-2}$ erhöht wurde, die Retention für saure und nicht-geladene Analyten graduell erhöht, aber die Retention für zwitterionische und insbesondere für basische Analyten niedriger. Die Zunahme der Retention mit zunehmender Ligandendichte ist im Einklang mit der Theorie des Verteilungsmechanismus, während ein Verlust der Retention möglicherweise durch die Anwesenheit von stark attraktiven Silanol-Analyte Interaktionen hervorgerufen wird. Saure Analyten zeigten eine stärkere Empfindlichkeit für die zunehmende Ligandendichte als nicht-geladene Analyten, was wahrscheinlich durch eine Verringerung der starken Repulsionen bei hoher Ligandendichte zwischen Silanol und Analyte erklärt werden kann. Dementsprechend sind silanophile Wechselwirkungen an dem HILIC Retentionsmechanismus beteiligt, vor allem für geladene und beladbare Verbindungen.

Mit Bezug auf den Effekt von Parametern der mobilen Phase auf Retention und Selektivität wurde eine weitere Studie durchgeführt. Hierbei wurde die Abhängigkeit von der Art der protischen Modifier in HILIC Trennungen untersucht, in denen der Wasseranteil in Acetonitril-reich Eluenten von verschiedenen Alkoholen (1,2-Ethandiol, Methanol, Ethanol) ausgetauscht wurde. Auf einer Reihe von Diol-Typ Säulen sowie auf einer nackten Silica stationäre Phase führten die nicht-wässrige aber polare Elutionsbedingungen zu einer stärkeren Retention von polaren Verbindungen. Das Ausmaß der Retentionszunahme war hierbei stark von der Polarität des eingesetzten Alkohol-Modifiers bestimmt und stieg tendenziell in der Reihenfolge 1,2-Ethandiol < Methanol < Ethanol an. Neben diesem Effekt auf die Retention, war chromatographische Selektivität

auch deutlich von der Art des Alkohol-Modifiers beeinflusst werden. Zum Beispiel führte unter isoelutropischen Bedingungen die mobile Phase mit Methanol als polarer Modifier zu einer umgekehrten Elutionsreihenfolge von Guanosin und Guanin im Vergleich zu wässrigen Eluent. Dies deutet das Auftreten der Solvent-spezifischen Effekte auf die Selektivität im HILIC Modus an.

Die Menge der protischen Modifier in der mobile Phase hatte auch erhebliche Effekte auf die Retention und Selektivität. Eine Verringerung der Menge an protischen Modifiern (< 40%, v/v) führte zu einer zunehmenden Retention für alle Analyten unter wässrigen HILIC und nicht-wässrigen HILIC Elutionsbedingungen. Das Ausmaß des Einflusses von einzelnen Analyten war nicht einheitlichen wodurch komplexe Effekte auf die Selektivität beobachtet wurden.

Neben dem eingeführten Parameter „Art und Menge des protischen Modifiers“ wurden in begleitenden Untersuchungen auch chromatographische Effekte bedingt durch (i) den pH-Wert des Puffers, (ii) Typ und Menge des Salzes, und (iii) die Säulentemperatur analysiert. Der pH-Wert (im Bereich von pH 3.0-8.0) hat keine großen Auswirkungen auf Retention und Selektivität für nicht-geladenen Analyten (die Variation der Retention war weniger als 10%). Für geladene und beladbare Verbindungen war die Änderung der Retention stärker. Unterschiedliche pH-Wert des Puffers führten zu verschiedenen Eluent $^{s}_{w}$ pH-Werten (gemessene pH-Wert nach Zugabe von organischem Lösungsmittel), die den Ionisationszustand des Silanols sowie des Analytes beeinflussen können.

Wenn die Salzkonzentration von Ammoniumacetat von 3.75 mM auf 30 mM (90% Acetonitril) erhöht wurde, wurden unter wässrigen Elutionsbedingungen alle saueren, schwach basischen und nicht geladenen Analyten länger retardiert. Hingegen wurde für starke Basen eine kleinere Retentionszeit beobachtet. Unter nicht-wässrigen HILIC Elutionsbedingungen wurden Zu- und Abnahme in der Retention für nicht-geladene Verbindungen beobachtet. Eine Zunahme der Retention mit zunehmender Salzkonzentration stimmte mit dem Ergebnis von einem hydrophilen Mechanismus überein, während eine Reduzierung der Retention bei einem von einer anziehenden ionische Wechselwirkung angetriebenen Mechanismus zu erwarten war. Weiterhin wurde gezeigt, dass die unterschiedlichen Salze mit den von ihnen hervorgerufenen

verschiedenen Eluent ${}^{s}_{w}$pH-Werte unterschiedliche Effekte auf Retention und Selektivität verursachen könnten.

Säulentemperatur ist ein weiterer wirksamer Parameter für Retention und Selektivität. Mit zunehmender Temperatur zeigten die meisten sauren und nicht-geladenen Analyten eine Abnahme der Retention unter wässrigen HILIC Elutionsbedingungen. Eine Zunahme der Retention wurde beobachtet für basische und zwitterionische Verbindungen. Das deutete stark auf unterschiedlichen Mechanismus für verschiedene Verbindungen hin. Unter nicht-wässrigen HILIC Elution wurden alle nicht-geladene Analyten kürzer retardiert bei hohen Temperatur, unabhängig von der Art des protischen Modifiers. Das Ausmaß der Abnahme von Retention war deutlich unterschiedlich für verschiedenen protischen Modifiern.

Neben dem Studium der Charakterisierung der chromatographischen Eigenschaften und der Erforschung der Retentionsmechanismen von nicht-geladenen Silica-basiert polaren stationären Phasen, ist die chromatographische Charakterisierung der RP/WAX Säulen im HILIC- und RP-Modus sowie die Untersuchung der entsprechenden HILIC Retentionsmechanismen auf solche geladenen Säulen ein weiterer Teil der vorliegenden Arbeit. Eine Reihe von RP/WAX Phasen mit unterschiedlichen RP/WAX Liganden, nämlich (i) N-undecanoyl 3-aminoquinuclidine, (ii) N-undecanoyl 3-aminotropane, (iii) N, N-dimethyl-N-10-undecanoyl -1,2-ethanediamine und (iv) N-butanoyl-(2S, 4S, 5R)-2-aminomethyl-5-[(2-octylthio) ethyl]-Chinuclidin wurden in unserer Gruppe entwickelt. In dieser Studie wurden sie mit kommerziellen Säulen (WAX-Typ, RP-Typ, RP/WAX-Typ, HILIC-Typ) verglichen. Im RP-Modus zeigten die entwickelten RP/WAX Phasen ausreichende Hydrophobizität ($k^{\text{pentylbenzol}}$ = 6.4~18.4) und Ionenaustausch-Fähigkeit mit Bezug auf die Retentionszeit der polaren Säure o,o-diethylthiophosphate (DETP) (k^{DETP} = 12.69~30.75). Andererseits eluierte DETP bei Totvolumen Zeit auf der single-Mode Synergi Fusion RP-Säule. Auf der WAX-Typ Phase wie z. B. Luna Amino konnte das DETP retardiert werden (k^{DETP} =4.39), aber keine CH_2-Inkrement Selektivität wurde auf dieser Säule beobachtet. Im Vergleich zu den Single-Mode stationären Phasen (RP-Phase und WAX-Phase), bot die RP/WAX Materialien einzigartige Retention und Selektivität Profile im RP-Modus.

Zusätzlich zeigte diese RP/WAX Phasen auch gute HILIC Trennungsfähigkeiten in Bezug auf Xanthin und Nukleosiden, während einige kritische Peak-Paare und auch

verschlechterte Peak-Shapes auf den typischen HILIC Säulen (TSK Gel Amid-80, ZIC-HILIC und Polysulfoethyl A) beobachtet wurden. Die multimodale Trennungseigenschaften der RP/WAX Phasen (hydrophob und ionisch) bieten die komplementäre Trennungsfähigkeiten für HILIC Applikationen. Im Hinblick auf den Retentionsmechanismus wurden elektrostatische Wechselwirkungen von den hydrophilen Interaktionen bei Adsorptions- und Verteilungsprozessen überlagert.

Außer der Ausweitung der Kenntnisse über die Phasen Evaluierung und über die Retentionsmechanismen im HILIC Modus, war der Versuch einer praktischen Anwendung der In-Hause entwickelten TGO Säule sowie einer Reihe von kommerziellen polaren Säulen ein weiterer Teil der Dissertation. *G. lucidum* ist ein Pilz der in der Traditionellen Chinesischen Medizin (TCM) Anwendung findet. Er ist bekannt wegen seiner wertvollen medizinischen und therapeutischen Funktionen. Aufgrund der Vielzahl von *Ganoderma* Spezies und der breiten geografischen Herkunft, ist es notwendig, eine geeignete Methode für Diskriminierung, Standardisierung und Qualitätskontrolle von *G. lucidum* zu etablieren. Die chromatographische Fingerprint-Analyse von *G. lucidum* wurde in HILIC-Modus (TGO Spalte, Luna HILIC, Polysulfoethyl A, TSK-Gel Amid-80 und ZIC-HILIC) und in RP-Modus (Zorbax SB-C18-Säule) durchgeführt. Die Motivation für das Kombinieren des HILIC- und des RP-Modus in dieser Studie was es, das Fenster der zugänglichen Polaritäten von Verbindungen zu erweitern und damit mehr Informationen für die Unterscheidung der *Ganoderma* Proben zu gewinnen. Die TGO-Phase zeigte gute Eignung zur chemometrischen Diskriminierung von einigen *Ganoderma* (G. lucidum, G. atrum, G. sinense, G. tsugae). Verschiedene HILIC Säulen lieferten ganz andere Peak-Muster, aber ihre Nutzbarkeit für die chemometrische Diskriminierung von *Ganoderma spp.* war sehr ähnlich. Dies bedeutet, dass die Art der polaren Gruppen in Ligand der stationärer Phase hatte nur geringe Auswirkungen auf ihre Eignung für das vorliegende Problem hat. Im HILIC Modus wurden falsche Klassifizierung der *Ganoderma spp.* für alle stationären Phasen beobachtet. Zum Beispiel wurden zwei von fünf *G. sinense* Proben auf der TGO-Phase und vier von fünf *G. sinense* auf TSK Gel Amid-80 und A-Säulen Polysulfoethyl Phase nicht richtig klassifiziert. Im RP-Modus wurden falsche Diskriminierungen für *G. atrum* und *G. sinense* Proben geliefert. Auf der anderen Seite zeigte eine Kombination der Daten des HILIC Modus und RP-Modus eine 100% richtig Unterscheidungsrate für die

Ganoderma spp.. Daher hat die multi-modale chromatographische Fingerprint-Analyse (HILIC & RP) Vorteil gegenüber der single-Mode-Analyse.

Folgende Schlussfolgerungen können aus den vorgelegten Ergebnissen gezogen werden. Die Phasenpolarität und die polaren Funktionalitäten in Liganden (Hydroxyl, Sulfoxid) wirken sich positiv auf die HILIC Eigenschaften der stationären Phase aus. Silanophile Interaktionen sind ein einflussreicher Faktor im HILIC Prozess, vor allem für geladene und beladbare Analyten. Neben der Menge des protischen Modifiers, dem pH-Wert des Puffers, der Art und Menge der Salze und der Säulentemperatur, ist die Art der protischen Modifiers ein entscheidender Parameter für die Methodenentwicklung und die Optimierung der HILIC Trennung für polare Verbindungen. Der HILIC Retentionsmechanismus zeigt komplexe Eigenschaften und es wird angenommen, dass die hydrophilen Interaktionen aus Verteilungs- und schwache Adsorptionsinteraktionen wie z.B. Wasserstoffbrücken, Dipol-Dipol-Wechselwirkungen induziert durch direkte Analyte-Liganden-Wechselwirkungen, Analyte-Solvatationsschicht auf der Oberfläche und/oder Analyte-Silanol bestehen. Bei geladenen stationären Phasen wurden die elektrostatischen Wechselwirkungen von hydrophilen Interaktionen überlagert.

Abstract

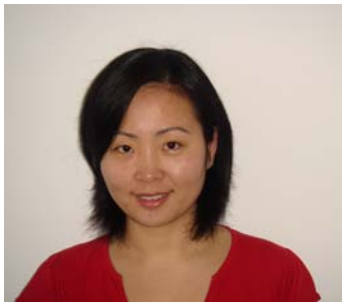
Four stationary phases having non-charged ligands with hydroxyl as well as sulfide- or sulfoxide-groups bonded to silica were prepared. 2-mercaptoethanol (ME phase) and 1-thioglycerol (TG phase) were immobilized on vinylised silica by radical addition. Subsequent on-phase oxidation of the embedded sulphide groups with hydrogen peroxide delivered sulfoxide-groups (MEO and TGO phases).

These new developed diol-type stationary phases offered HILIC chromatographic selectivity towards the separation of nucleosides, nucleobases and water soluble vitamins. Higher Phase polarity caused a stronger retention of polar compounds and sulfoxide functionality in the ligand was especially useful to change HILIC characterisation of phases. Compared to commercial diol-type HILIC packings complementary selectivity was found which may be an indication for different retention mechanisms being at work. The developed diol-type phases delivered also noticeable CH_2-increment selectivity (multimodal separation capability). Silanol activity had definite effects on retention and selectivity in the HILIC elution mode and the extent of response was depending on the type of analytes (acids, bases, non-charged). In the HILIC mode, silanophilic interactions may become the main retention mechanism on silica-based polar phases, especially for charged and chargeable compounds.

The type of protic modifier (water, 1,2-ethanediol, methanol, ethanol) had significant effect on HILIC retention and the polarity of these protic modifiers adopted a primary role in determining the extent of retention and, generally, increased retention was found in the order water < 1,2-ethanediol < methanol < ethanol. Besides the effect on retention, chromatographic selectivity was also affected by the type of alcohol modifier and under isoelutropic conditions compared to an aqueous HILIC eluent the selectivity can be changed up to an inverted elution order. Additionally, the amount of organic solvent, the nature and amount of salt, pH value and column temperature were all effective to optimise HILIC separations.

The retention mechanisms were quite complex in HILIC. It was supposed that the hydrophilic interactions were composed of partitioning and weak adsorption interactions which could be developed by direct solute-ligand interactions, solute-solvent shell on the surface, and/or solute-silanol interactions. For charged packings, electrostatic interactions (attractive or repulsive) can be easily superimposed on the hydrophilic interactions.

Curriculums Vitae



Name:	**Wu Junyan**
Akademischer Grad:	Mag. rer. nat.
Geburtsdatum:	16.02.1978
Geburtsort:	Zhejiang
Staatsbürgerschaft:	Volksrepublik China
Adresse:	Silbergraben 32
	99097 Erfurt – Deutschland
Familienstand:	Verheiratet
Telefon:	+43-676-9607828
E-mail:	wujunyan0216@hotmail.com

Ausbildung

03/2007 – 09/2009	**Doktoratsstudium**, Dissertation Chemie (Universität Wien, Österreich)
10/2002 – 02/2006	**Diplomstudium Chemie** (Universität Wien, Österreich)
03/2002 – 07/2002	**Vorstudienlehrgang Deutsch** (Universität Wien, Österreich)
09/1997 – 06/2001	**Diplomstudium Ingenieur Chemie** (Zhejiang Technologische Universität, Volksrepublik China)

Sprachenkenntnisse

Deutsch in Wort und Schrift

Englisch in Wort und Schrift

Chinesisch - Muttersprache

Verzeichnis der wissenschaftlichen Aktivität

Diplomarbeit Ingenieur Chemie (02/2001 – 06/2001)

Titel:" *"Mathematisches Korrelation des Einfluss von Metallsedimente auf die Eigenschaft*

des Krackenkatalysators"

Betreuer: O.Uni.-Prof. Dr. Jie Ren; Institut für Chemieingenieur und Technologie, Zhejiang Technologische Universität, Volksrepublik China

Diplomarbeit Chemie (03/2006 – 12/2006)

Titel:*" Synthese und Evaluierung von neuen Kieselgelmaterialien mit verschiedener Funktionalität für die Chromatographie "*

Betreuer: Prof. Dr. Wolfgang Lindner

Christian Doppler Labor für Molecular Recognition Materials, Institut für Analytische Chemie und Lebensmittelchemie, Universität Wien, Österreich

Dissertation Chemie(03/2007 – laufend)

Titel:"*Development and evaluation of new silica based polar and / or mixed modal stationary phases for HPLC"*

Betreuer: Prof. Dr. Wolfgang Lindner

Christian Doppler Labor für Molecular Recognition Materials, Institut für Analytische Chemie und Lebensmittelchemie, Universität Wien, Österreich

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Separation properties of novel polar stationary phases in hydrophilic interaction (aqueous and non-aqueous) and reversed-phase liquid chromatography modes

Anakon 2009, 17. - 20. März 2009, Berlin, Deutschland

(2) Junyan Wu, Wolfgang Bicker and Wolfgang Lindner

Contributions of surface silanols to retention and selectivity properties of bonded silica packings in hydrophilic interaction chromatography

34th International Symposium on High Performance Liquid Phase Separations and Related Techniques (**HPLC 2009**)**,** 28. June – 2. July, 2009, Dresden, Deutschland

(3) Junyan Wu, Yi Chen, Wolfgang Bicker, Wolfgang Lindner

Liquid chromatographic fingerprint analysis of Ganoderma spp. extracts in hydrophilic interaction and reversed-phase elution modes using UV and evaporative light-scattering detection

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Selectivity in aqueous- and nonaqueous-HILIC mode for polar analytes on silica-based non-charged polar stationary phase

Junganalytiker(Innen) Forum, Wien, Österreich**, 30. - 31 Mai 2008)**