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On the characterization of hydrophilic interaction liquid chromatography stationary phases

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ABSTRACT

It is generally accepted that partitioning is the main retention mechanism involved in hydrophilic interaction liquid chromatography (HILIC). However, secondary interactions, such as dipole-dipole and ion-exchange, can play an important role in the separation, leading to changes in selectivity. It is therefore essential to understand and characterize secondary retention mechanisms in order to make a judicious selection of the column. This article presents the results of a HILIC chromatographic characterization regime carried out on ten silica-based columns, including unmodified silica, amino, diol, ion exchanger and zwitterionic materials, and a Porous graphitic Carbon (PGC) column. Based on the HILIC characterization scheme developed by Tanaka's group, the testing describes the structure-selectivity relationships between analytes and stationary phases. The retention properties investigated include the degree of hydrophilicity, the selectivity for hydrophilic and hydrophobic groups, positional selectivity, the anion and cation exchange properties and the evaluation of the stationary phase pH. The data were summarized as radar plots, which proved to be useful to distinguish the overall selectivity of the HILIC stationary phases and ultimately can be used as a column selection tool in HILIC method development.

KEYWORDS: hydrophilic interaction liquid chromatography, chromatographic characterization, selectivity, retention factor, column classification, polar solutes

INTRODUCTION

The tremendous popularity of reversed-phase liquid chromatography, (RPLC) has been due to its suitability for a wide variety of analyte classes, especially in the bioanalytical and pharmaceutical arenas [1]. In recent years, however, the development of an increasing number of hydrophilic drugs has resulted in other techniques growing in popularity, since hydrophilic compounds cannot be sufficiently retained by RP packing materials [2]. One of the most successful approaches to the retention of polar compounds is hydrophilic interaction liquid chromatography (HILIC), which has been described as 'reversed RP' [1]. In HILIC the stationary phase is polar and the aqueous portion of the mobile phase acts as the stronger solvent.

The acronym HILIC was first introduced in 1990, by Alpert [3]; however, as a separation technique it had been used for several decades. As outlined in the comprehensive review by Hemström and Irgum [1], the HILIC mode of separation can be traced back to 1951, when Gregor *et al.* [4] described a water-enriched layer on an ion-exchange resin surface. The following year, Samuelson and Sjöström analysed monosaccharides on an ion-exchange column [5]. Then, in 1954, Rückert and Samuelson [6] suggested the possibility that a stagnant water layer could be responsible for the uptake of analytes. Several years later, in 1975, the analysis of sugars was accomplished on amino columns [7, 8]. Nowadays, the existence of a water-enriched layer on the polar stationary phase, combined with a partitioning equilibrium of analytes are the fundamentals for the accepted HILIC mechanism [1, 3].

In spite of its early beginnings, HILIC did not become widely recognised as a distinct chromatographic mode until it was 'rediscovered' by the scientific communities in the early 2000's [9]. The rising popularity of HILIC coincided with a wider availability of specifically designed HILIC stationary phases with diverse functionalities, which could offer different selectivity and higher retentions for polar compounds [9]. The fact that the solvents used in HILIC are mass spectrometry compatible and their high concentrations increase ESI-MS sensitivity further contributed to a widening of the range of HILIC applications [9].

What follows is a review of the most widely used HILIC materials. While it is not intended to be comprehensive, the intention is to facilitate the understanding of the differences in retention and selectivity of various materials and to highlight the need to categorize these materials.

Prior to the early 2000's, most HILIC separations were carried out on normal phase columns, such as amino, cyano and bare silica [9]. Silica continues to be a popular phase for HILIC, although in normal phase it has always been problematic [10]. McCalley discussed reproducible HILIC retention of analytes on silica substrates and he envisaged the presence of significant levels of water as being the reason for reproducible elution behaviours [10]. Several column manufacturers have developed silica columns specifically intended for HILIC, which are packed and stored in aqueous/organic solvents. Silica materials have also become available in sub-2 µm particles, in superficially porous particles and monolithic; their retention mechanisms and their efficiencies have been assessed in several HILIC reviews [11-13]. Cyano phases find limited applications in HILIC, as insufficient retention of most polar compounds is generally experienced. This is due to the fact that cyano groups do not have hydrogen bond donor capabilities and therefore are not very hydrophilic [14]. On the other hand, diol phases have found more HILIC applications, possibly due to their higher degree of hydrophilicity, which affords sufficient retentivity [9]. Amino materials are widely used for HILIC separations, especially for carbohydrate analysis [1]. Recently, however, Ikegami *et al.* commented on their potential irreversible adsorption of analytes (or at least for reducing sugars), due to the reactive nature of amino functionalities, and suggested that this could be one reason for their declining use in sugar analysis [13].

Amide-bonded silica phases are the most popular choice in HILIC. The amide group is less reactive and less basic than the amine group, so retention is less dependent on the mobile phase pH and less prone to irreversible adsorption [1]. Amide columns have also demonstrated reproducibility and stability [15]. Ikegami *et al.* reviewed HILIC applications which employ amide columns (among others) [13], as a follow-up to Hemström and Irgum's report [1]. Both evaluations covered the ongoing discussion on the separation mechanisms involved in HILIC and the main bonding procedures. Zwitterionic phases are widely used for HILIC separations. Irgum *et al.* originally introduced the sulfoalkylbetaine zwitterionic functionality to polymer supports to prepare ion-exchange materials for inorganic compounds analysis [16, 17] and proteins analysis [18]. Subsequently, a similar functionality was immobilized on silica substrates [1]. The sulfobetaine zwitterion has both positive (quaternary ammonium) and negative (sulfonic acid) groups in a 1:1 ratio [19], so that the net surface charge is zero. It has been pointed out, though, that the negative charge of the sulfonic acid at the distal end of the phase may introduce electrostatic interactions with charged analytes [20, 21]. Irgum and his group have discussed a new type of zwitterionic phase, with a phosphorylcholine group grafted on a polymeric substrate [22]. This material has a positively charged ammonium group at its distal end.

As already discussed, HILIC can be described as a variation of reversed phase chromatography performed using a polar stationary phase. The mobile phase employed is highly organic in nature (>70% solvent, typically acetonitrile) containing also a small percentage of aqueous solvent/buffer or other polar solvent. The water/polar solvent forms an aqueous-rich sub-layer adsorbed to the polar surface of the stationary phase into which analytes partition. The resulting retention order is roughly the opposite of the order analytes elute from a reversed phase column [3]. McCalley and Neue demonstrated the existence of the water-rich layer on the silica surface under the typical HILIC

conditions, using toluene and benzene as test probes [23]; they were also able to observe that the water-rich layer increased in thickness as the aqueous content in the mobile phase increased up to 30%. Subsequently McCalley investigated the retention behaviour of a mixture of neutral, acidic and basic compounds on several HILIC materials [24]; the study showed the existence of a very complex mechanism, consisting of a combination of hydrophilic partitioning interaction, adsorption, ionic interactions and even hydrophobic interactions. Liang *et al.* proposed a HILIC retention model, where the predominant mechanism depends on the analyte characteristics, the mobile phase composition and the nature of the stationary phase [25].

Although it was demonstrated that the organic modifier/aqueous ratio is the predominant factor in providing the necessary separation selectivity in HILIC [2], the choice of stationary phase is also very important. Chirita *et al.* suggested a column selection scheme and applied it to neurotransmitters analysis [26]. They advocated choosing HILIC columns according to the nature of the interactions between analyte and stationary phase. Fine-tuning the separation by optimising the organic solvent content, the buffer concentration and the mobile phase pH would follow in the decision tree. This approach to HILIC method development highlights the importance of column selection. Given the fact that the stationary phases used in HILIC are quite diverse (Hemström and Irgum described more than forty separation materials used for HILIC applications [1]), choosing the optimal column can be very challenging.

Systematic studies of HILIC materials chemistries and the roles of their functional groups have been limited. A combination of these factors has lead to confusion and difficulties during HILIC column selection for method development. Pontén recently commented on the fact that users are under the impression that 'HILIC columns' are interchangeable [27], despite the difference in the chemical structure of the various HILIC stationary phases. This belief has probably been encouraged by the increasing interest and demand for HILIC methodologies which in turn resulted in an increase in the 'HILIC' branded products. HILIC column comparison studies have been undertaken in the last few years; however, these studies concerned specific classes of compounds and probed specific interaction modes. For example, Guo and Gaiki [9] have reviewed the retention and selectivity of several HILIC materials and classified them into three main groups: charged, neutral and zwitterionic phases. It was argued that this classification depended on the nature of the test compounds and did not consider that silica-based neutral materials could effectively have cation exchange interactions due to free silanols [9]. The presence of ion exchange contribution, independently from the nature of the stationary phase but brought about by the basic nature of the analyte was demonstrated by McCalley [24], whom also showed that for the more hydrophobic bases the extent of ion exchange contribution increased. Other HILIC characterization studies have been reported. Lämmerhofer *et al.* [28] used xanthines, nucleosides and water soluble vitamins as test samples on bare silica, amino-, amide-, zwitterionic and sulfonate-bonded phases. Chauve *et al.* [29] suggested a test scheme for bare silica materials comprising 15 test compounds, including saccharides, nucleobases and amino acids. Marrubini *et al.* [30] assessed amide-bonded and zwitterionic phases, using nucleic bases and nucleosides.

However, none of these studies discussed partial structure selectivity. More recently, both Tanaka's [31] and Irgum's [32] groups, independently suggested two comprehensive and seminal characterisation studies to classify HILIC columns and investigate HILIC retention mechanisms, focusing on specific interactions.

Irgum and co-workers designed a method based on selectivity factors for pairs of similar chemical compounds, one with properties promoting the particular interaction being assessed and the second one lacking such properties. The HILIC interactions characterised by Irgum and his group were: hydrophilic, hydrophobic, electrostatic, hydrogen bonding, dipole-dipole, π-π interaction and shape selectivity [32].

Tanaka's group followed a similar approach but using different test compounds in their HILIC characterization work [31]. In addition to retention characteristics and selectivity, they examined separation efficiency and peak resolution. The method they suggested could probe specific secondary interactions, namely: degree of

hydrophilicity, selectivity for hydrophilic-hydrophobic groups, selectivity for positional and configurational isomers, evaluation of electrostatic interactions and evaluation of the acidic-basic nature of the stationary phases. The data from this study showed structure-selectivity relationship for the various HILIC phases and represent a good approach to HILIC column selection for when targeting separations whose analytes possess some of the same structural characteristics. Tanaka and co-workers summarised the column properties in radar plots, whose shapes help to identify two main groups of stationary phases: (i) phases containing sulfonates, amides and zwitterionic groups, which demonstrated higher selectivity and retentivity for the test compounds; (ii) phases containing hydroxy and amino groups and no functionalities, which showed relatively limited retentivity and selectivity.

This testing scheme was applied in our laboratory, for examining columns with the following chemistries: bare silica, zwitterionic-, amino-, amide-, mixed-mode diol-, mixed-mode RP/anion-exchange/cation exchange (** in Table 1, Nanopolymer Silica Hybrid, NSH)- phases and a silica phase covalently modified with an hydrophilic group and an anion-exchanger (^ in Table 1). A Porous Graphitic Carbon (PGC) material was included in the study. PGC is made of spherical, fully porous particles, which at molecular level are made up of graphitic layers of hexagonally arranged carbon atoms, with no functional groups on the surface. Although its surface is hydrophobic, its retention capabilities for polar analytes have been demonstrated in both typical RP and HILIC mobile phase conditions [33, 34].

MATERIALS AND METHODS

Chemicals and reagents

HPLC grade acetonitrile, water, acetone and toluene, analytical grade ammonium acetate and Optima grade acetic acid were obtained from Fisher Scientific (Loughborough, UK). Uridine 5-methyluridine, 2'-deoxyuridine, adenosine, vidarabine, 2'-deoxyguanosine, 3'-deoxyguanosine, uracil, sodium p-toluenesulfonate, N,N,N-trimethylphenylammonium chloride, theobromine and theophylline were purchased from Sigma-Aldrich (Poole, UK).

Chromatogaphic tests

The chromatographic conditions were kept unaltered throughout the comparison study; the mobile phase consisted of 90:10 (v/v) acetonitrile:ammonium acetate (20 mM on the column, pH 4.7). The flow rate was fixed at 0.5 mL/min. UV detection was carried out at 254 nm. The injection volume was of 5 µL. All runs were done with active thermostatting of the columns

Table 1. Specifications of the HILIC columns used. *PGC: Porous Graphitic Carbon. **NSH: Nanopolymer Silica Hybrid.

Column name	Phase type	Column dimension (mm)	Surface area (m^2/g)	Pore size (Å)
Syncronis HILIC (5 µm)	Zwitterion	100 x 4.6	320	100
Hypersil GOLD HILIC (5 µm)	Polyethyleneimine	100 x 4.6	220	175
Hypersil GOLD Silica (5 µm)	Unbonded Silica	100 x 4.6	220	175
Hypersil GOLD Silica (1.9 µm)	Unbonded Silica	100 x 4.6	220	175
Syncronis Silica (5 µm)	Unbonded Silica	100 x 4.6	320	100
Accucore HILIC (2.6 µm)	Unbonded Silica	100 x 4.6	130	80
Acclaim Mixed Mode HILIC-1 (5 µm)	Mixed Mode Diol	150 x 4.6	300	120
Acclaim HILIC-10 (3 µm)	Proprietary^	150 x 4.6	300	120
Acclaim Trinity P1 (3 µm)	NSH**	150 x 3.0	100	300
Experimental HILIC (3 µm)	Polyacrylamide	150 x 3.0	100	90
Hypercarb (5 µm)	PGC*	100 x 4.6	120	250

at 30°C. The columns assessed in this study are reported in Table 1. They cover a range of surface chemistry and physical properties (with regards to particle size and pore size). All the columns were from Thermo Scientific (Runcorn, UK).

Retention factors were determined as the average of six injections and toluene was used as unretained marker (t_0). Acetone was used as t_0 marker on Hypercarb, since toluene is highly retained by PGC under the chromatographic conditions used in this study.

Instrument

Chromatographic experiments were carried out on two instruments: an HP 1100 HPLC system, (Agilent Technology Waldbronn, Germany) and an Accela UHPLC system (Thermo Scientific, San Jose, USA). ChemStation Software Rev. A. 10. 02 (Agilent, Waldbronn, Germany) and ChromQuest 5.0 (Thermo Scientific, San Jose, USA) were used to control the HPLC and UHPLC systems, respectively and to process the chromatographic data.

Test mixtures

All the stock solutions for the individual test probes were prepared in mobile phase at 1 mg/mL. The test mixtures comprised selected pairs of compounds that were expected to vary in their interactions with the stationary phases, plus the t_0 marker. A total of seven test mixtures were prepared and they were: test mixture 1: t_0, uridine (U), 5-methyluridine (5MU); test mixture 2: t_0, uridine, 2'-deoxyuridine (2dU); test mixture 3: t_0, adenosine (A), vidarabine (V); test mixture 4: t_0, 2'-deoxyguanosine (2dG), 3'-deoxyguanosine (3dG); test mixture 5: t_0, uracil (Ur), sodium p-toluenesulfonate (SPTS); test mixture 6: t_0, uracil, N,N,N-trimethylphenylammonium chloride (TMPAC); test mixture 7: t_0, theobromine (Tb), theophylline (Tp). The chemical structures of the test compounds used in this study, together with their physiochemical properties are given in Table 2.

RESULTS

The degree of surface coverage of silica by hydrophobic groups is a useful parameter in both

Table 2. Structures of test solutes and their physiochemical properties (pKa and Log D values obtained from www.chemspider.com).

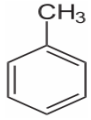
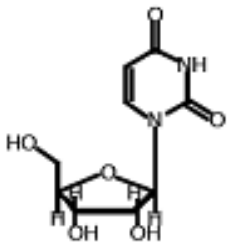
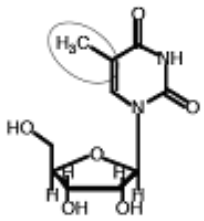
Chromatographic probes	Molecular structure	Variable	pKa	Log D	Test mixture
Toluene		t_0 marker	41	2.72	all
Uridine		Hydrophilic/ hydrophilic interaction	12.6	-1.58	1+2
5-Methyluridine		Hydrophobic interaction	12.0	-1.02	1

Table 2 continued..

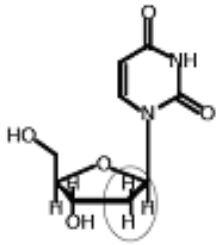
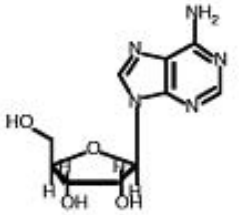
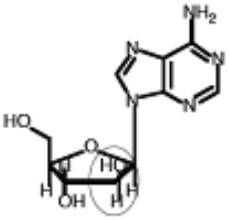
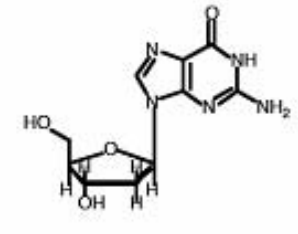
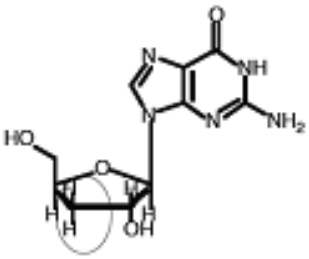
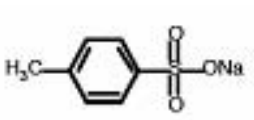
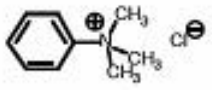
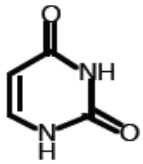
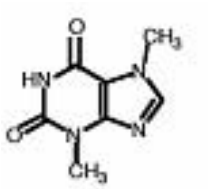
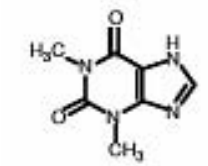
2'-Deoxyuridine		Hydrophilic interaction	13.9	-1.26	2
Adenosine		Configurational isomers selectivity	13.9	-1.03	3
Vidarabine		Configurational isomers selectivity	13.9	-1.02	3
2'-Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	4
3'-Deoxyguanosine		Positional isomers selectivity	13.5	-1.14	4
Sodium p-toluenesulfonate		Anion exchange selectivity	-2.8	0.88	5

Table 2 continued..

N,N,N-trimethylphenylammonium chloride		Cation exchange selectivity		-2.31	6
Uracil		Hydrophilic interaction	13.8	-1.08	5.6
Theobromine		Acidic-basic nature of stationary phase	10	-1.06	7
Theophylline		Acidic-basic nature of stationary phase	8.6	-2.51	7
Acetone		t_0 (for Hypercarb)	24	-0.04	all

RPLC [34] and HILIC because it provides an indication of the degree of hydrophobic interaction between the stationary phase and the test compounds. It can be measured from the selectivity for a methylene group, α (CH2). In this study α (CH2) was obtained from a comparison of $k_{uridine}$ (retention factor for uridine) and $k_{5\text{-methyluridine}}$ (retention factor for 5-methyluridine). Uridine and 5-methyluridine were chosen as the α (CH2) probe pair since they are polar enough to afford retention in HILIC. Figure 1 shows chromatograms for five representative columns.

From Figure 1 it can be seen that uridine is more retained than 5-methyluridine, which reflects the fact that uridine is more hydrophilic than 5 MU. With Hypercarb, the more hydrophilic uridine elutes first, indicating that polar interactions are less dominant on PGC.

The degree of hydrophilic interaction between the stationary phase and the test compounds was assessed using the selectivity for an hydroxy group, α (OH). In this study α (OH) was obtained from a comparison of $k_{uridine}$ and $k_{2'\text{-deoxyuridine}}$. Figure 2 shows chromatograms for five representative columns.

From Figure 2 it can be seen that uridine is more retained than 2'-deoxyuridine; this reflects the fact that uridine is more hydrophilic than 2 dU. Hypercarb does not discriminate between U and 2 dU, under these experimental conditions. Average α (CH2), α (OH) and k uridine values are summarized in Table 3 and the normalized data are reported in Figure 7.

An important property that HILIC must be able to afford is the capability to separate structural isomers and configurational isomers, typically found in saccharides and peptides. The selectivity of configurational isomers, α (V/A) - obtained from a comparison of $k_{vidarabine}$ and $k_{adenosine}$ - and positional (regio) isomers, α (2dG/3dG) - calculated

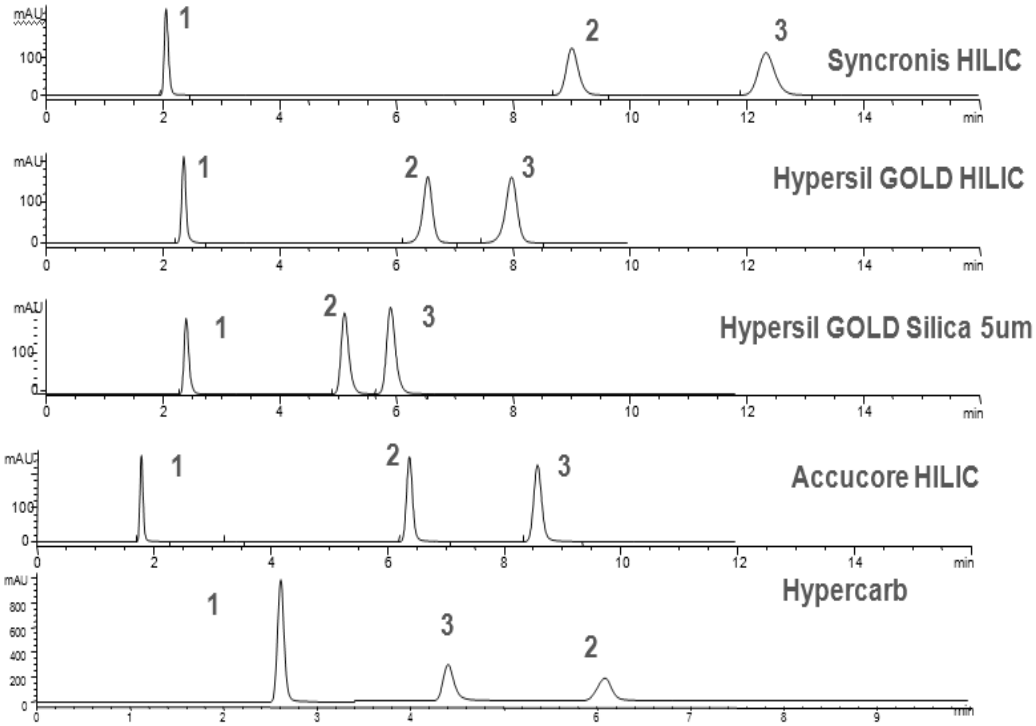


Figure 1. Chromatograms for α (CH2) test. Analyte: 1) toluene (acetone on Hypercarb); 2) 5-methyluridine; 3) uridine.

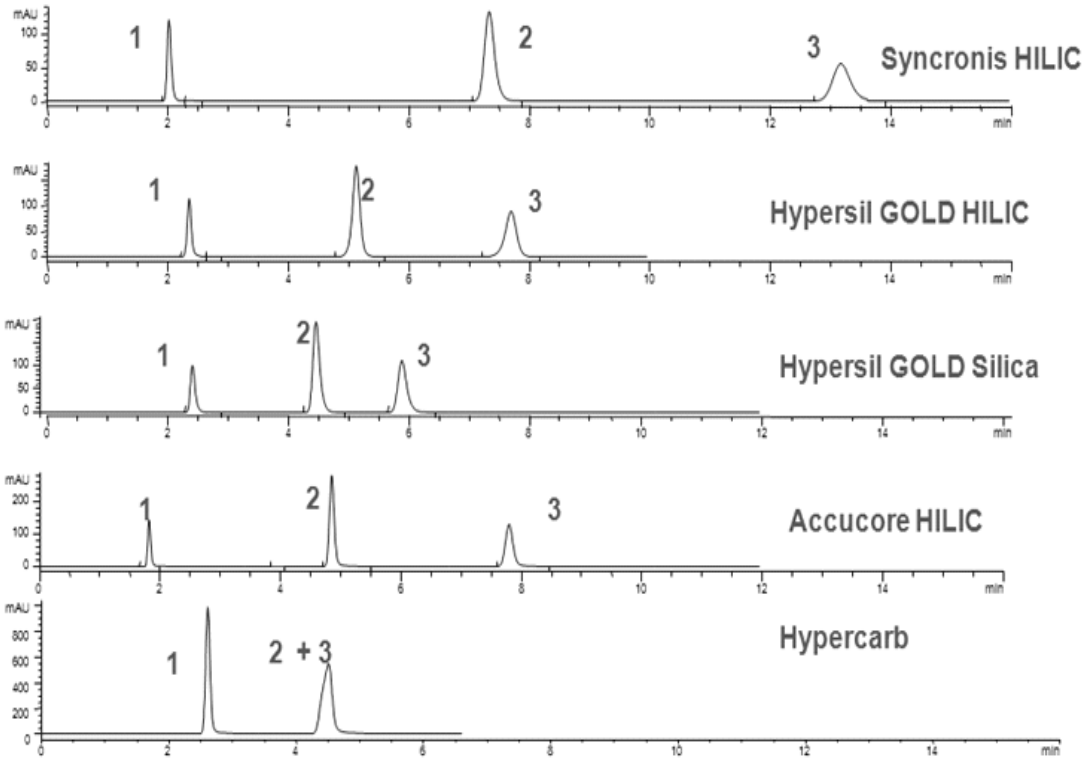


Figure 2. Chromatograms for α (OH) test. Analyte: 1) toluene (acetone on Hypercarb); 2) 2'-deoxyuridine; 3) uridine.

Table 3. Separation factors for methylene α (CH2) and hydroxy α (OH) groups and retention factor for uridine.

Column name	α (CH2)	α (OH)	k uridine
Syncronis HILIC (5 µm)	1.477	2.090	5.053
Hypersil GOLD HILIC (5 µm)	1.330	1.931	2.278
Hypersil GOLD Silica (5 µm)	1.291	1.697	1.377
Hypersil GOLD Silica (1.9 µm)	1.253	1.579	1.340
Syncronis Silica (5 µm)	1.302	1.518	3.152
Accucore HILIC (2.6 µm)	1.473	1.942	3.753
Acclaim Mixed Mode HILIC-1 (5 µm)	1.000	1.000	0.112
Acclaim HILIC-10 (3 µm)	1.117	1.521	1.836
Acclaim Trinity P1 (3 µm)	1.226	1.828	0.869
Experimental HILIC (3 µm)	1.530	2.182	3.513
Hypercarb (5 µm)	0.526	1.000	4.610

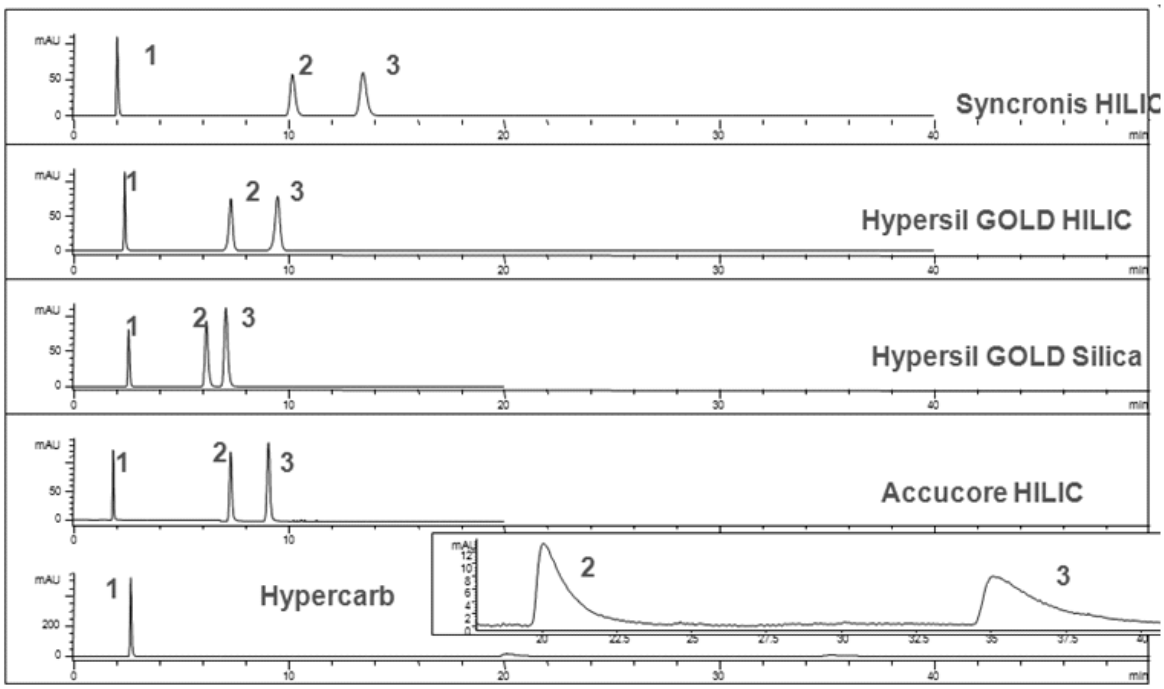


Figure 3. Chromatograms for α (V/A) test. Analyte: 1) toluene (acetone on Hypercarb); 2) adenosine; 3) vidarabine.

from the k_{2dG}/k_{3dG} ratio - were investigated. These selectivity values also reflect shape selectivity [31]. The selected chromatograms for the configurational isomers A and V and for the positional isomers 2 dG and 3 dG are reported in Figures 3 and 4, respectively.

The configurational isomers are separated by the columns under investigation, with vidarabine being more retained than adenosine. The two regio isomers are separated by the columns under investigation, although baseline resolution is not always achieved. Table 4 summarizes the mean

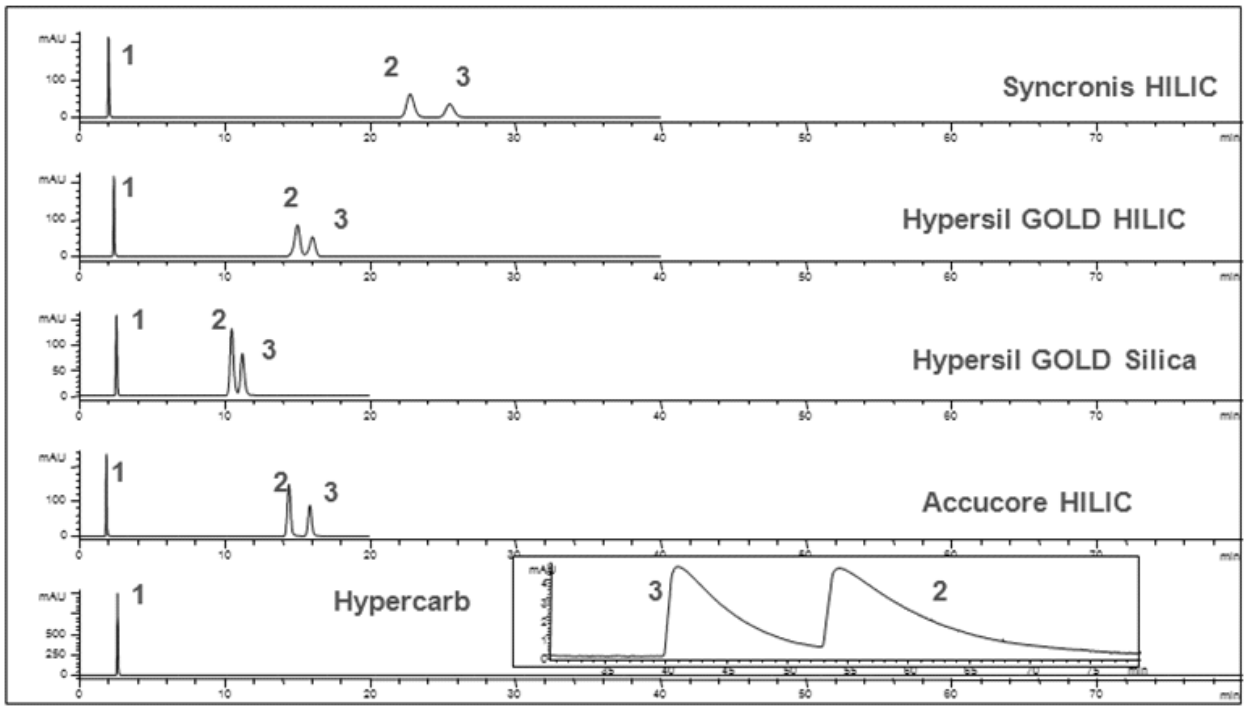


Figure 4. Chromatograms for α (2dG/3dG) test. Analyte: 1) toluene (acetone on Hypercarb); 2) 3'-deoxyguanosine; 3) 2'-deoxyguanosine.

Table 4. Separation factors for configurational isomers α (V/A) and positional isomers α (2dG/3dG).

Column name	α (V/A)	α (2dG/3dG)
Syncronis HILIC (5 µm)	1.403	1.129
Hypersil GOLD HILIC (5 µm)	1.444	1.082
Hypersil GOLD Silica (5 µm)	1.255	1.092
Hypersil GOLD Silica (1.9 µm)	1.214	1.092
Syncronis Silica (5 µm)	1.270	1.100
Accucore HILIC (2.6 µm)	1.327	1.114
Acclaim Mixed Mode HILIC-1 (5 µm)	1.000	1.102
Acclaim HILIC-10 (3 µm)	1.222	0.963
Acclaim Trinity P1 (3 µm)	1.409	1.023
Experimental HILIC (3 µm)	1.336	1.111
Hypercarb (5 µm)	1.863	0.744

α (V/A) and α (2dG/3dG) values for the stationary phases tested. The corresponding normalized data are illustrated in Figure 7.

Ion-exchange interactions can be influential in HILIC, leading to drastic changes in selectivity, particularly when separating ionic species [21]. To evaluate the degree of ion exchange nature of the stationary phases a relatively hydrophobic organic anion, sodium p-toluenesulfonate (SPTS) and a relatively hydrophobic organic cation, N,N,N-trimethylphenylammoniumchloride (TMPAC)

were chosen. It is reasonable to postulate that these compounds would also be retained by hydrophilic interactions [31], so the retention factors k_{SPTS} and k_{TMPAC} were divided by k_{Uracil} to account (at least partially) for the hydrophilic interaction contribution. The chromatography for both the anion and cation exchange interactions on the selected columns is shown in Figure 5 and Figure 6, respectively. The resulting mean separation factors, α (AX) and α (CX) for the stationary phases tested are reported in Table 5; the normalized values are plotted in Figure 7.

Figure 5 shows that, for some materials SPTS elutes before uracil, the exception being Hypersil GOLD HILIC, where SPTS elutes after uracil (this is also the case for Acclaim Trinity P1, although its chromatogram is not shown). Anion exchange selectivity varied also for Acclaim HILIC-1, where SPTS was not retained and it eluted before toluene and for Acclaim HILIC-10, where SPTS co-eluted with uracil (chromatography not shown).

From Figure 6 it can be seen that for some materials TMPAC elutes after uracil, apart from Hypercarb, where it is not retained, eluting before acetone (t_0 marker for Hypercarb) and Hypersil GOLD HILIC, where it elutes before uracil. Different cation exchange selelctivity was also exhibited by Acclaim HILIC-1 and Acclaim Trinity P1, with TMPAC co-eluting with uracil (chromatography not shown).

An aspect that has not received much attention in HILIC is the pH on the surface of the stationary phase [31]. Since many compounds analyzed in HILIC have ionizable functional groups, knowing the acid-base properties of the stationary phase is important for controlling the separation [31]. Xanthine derivatives have been used as test samples in HILIC; the pKa values for theophylline and theobromine have been reported as pKa = 8.6 and pKa = 10 respectively, so theobromine is more basic than theophylline [28]. The selectivity values, k_{Tb}/k_{Tp} obtained during our investigation are reported in Table 6; the normalized values are shown in Figure 7.

DISCUSSION

The data sets for the separation factors generated in this study highlighted important retention

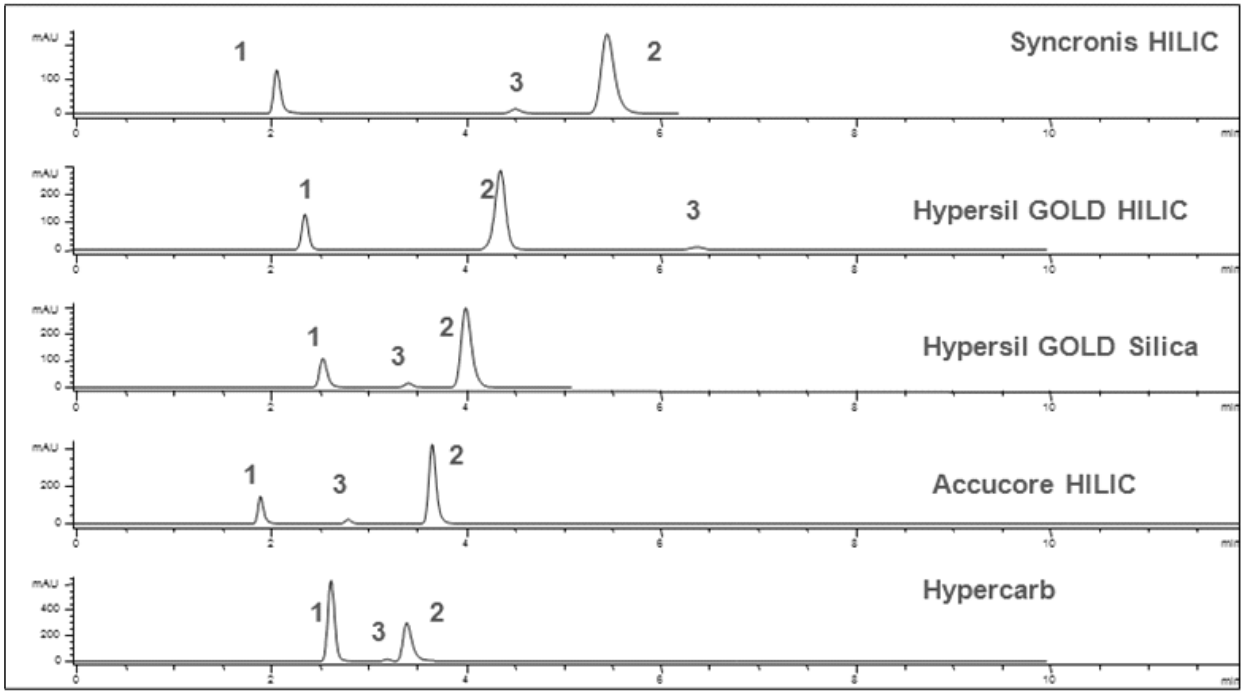


Figure 5. Chromatograms for α (AX) test. Analyte: 1) toluene (acetone on Hypercarb); 2) uracil; 3) sodium p-toleuenesulfonate, SPTS.

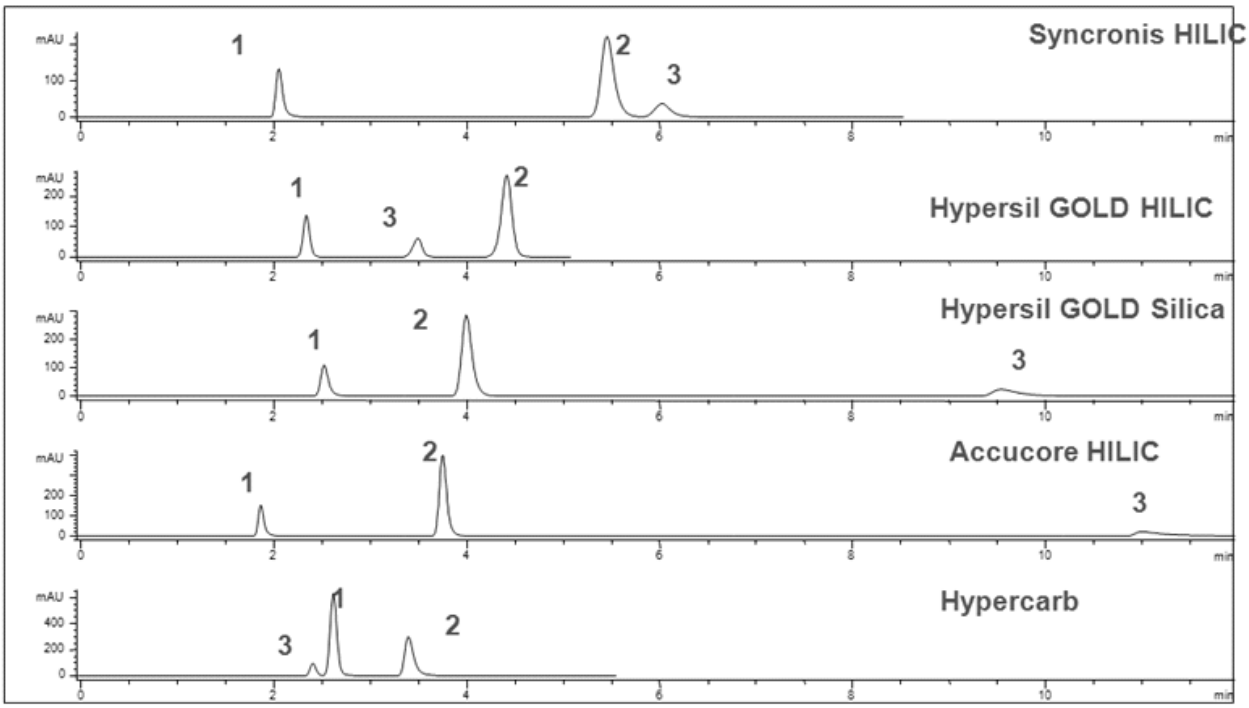


Figure 6. Chromatograms for α (CX) test. Analyte: 1) toluene (acetone on Hypercarb); 2) uracil; 3) N,N,N-trimethylphenylammoniumchloride, TMPAC.

Table 5. Separation factors for α (AX) and α (CX).

Column name	**α (AX)**	**α (CX)**
Syncronis HILIC (5 µm)	0.723	1.115
Hypersil GOLD HILIC (5 µm)	1.878	0.554
Hypersil GOLD Silica (5 µm)	0.609	4.832
Hypersil GOLD Silica (1.9 µm)	0.549	5.951
Syncronis Silica (5 µm)	0.581	5.614
Accucore HILIC (2.6 µm)	0.521	3.992
Acclaim Mixed Mode HILIC-1 (5 µm)	-	0.000
Acclaim HILIC-10 (3 µm)	1.000	1.919
Acclaim Trinity P1 (3 µm)	9.241	1.000
Experimental HILIC (3 µm)	0.454	1.660
Hypercarb (5 µm)	0.738	-

characteristics and differences (Table 3-6). Stationary phase characteristics have been visually illustrated by radar graphs [31, 35], which allow to express multi-dimensional data in a two-dimensional format and ultimately allow to visually assess and compare columns. The separation factors obtained in the course of this assessment were therefore arranged in radar plots, which are shown in Figure 7. In order to show the differences in selectivity all the α values were normalized to the largest value. Therefore, the full axis on the radar plot has a maximum value of 1. The following discussion will concern both tabulated data and their corresponding graphical representations.

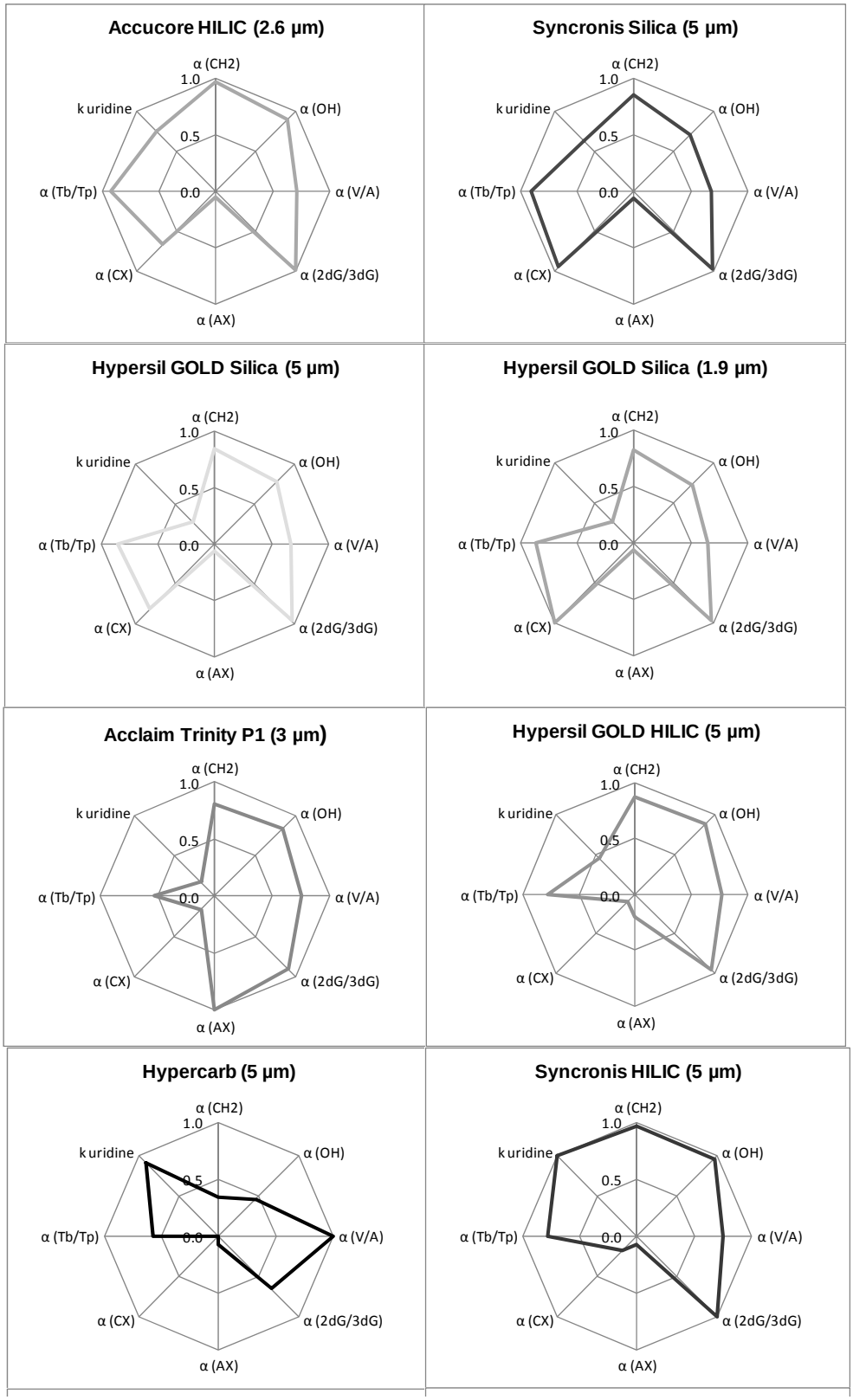

Accucore HILIC (2.6 µm)
Syncronis Silica (5 µm)
Hypersil GOLD Silica (5 µm)
Hypersil GOLD Silica (1.9 µm)
Acclaim Trinity P1 (3 µm)
Hypersil GOLD HILIC (5 µm)
Hypercarb (5 µm)
Syncronis HILIC (5 µm)
α (CH2)
α (OH)
α (V/A)
α (2dG/3dG)
α (AX)
α (CX)
α (Tb/Tp)
k uridine
1.0
0.5
0.0

Figure 7

Figure 7 continued..

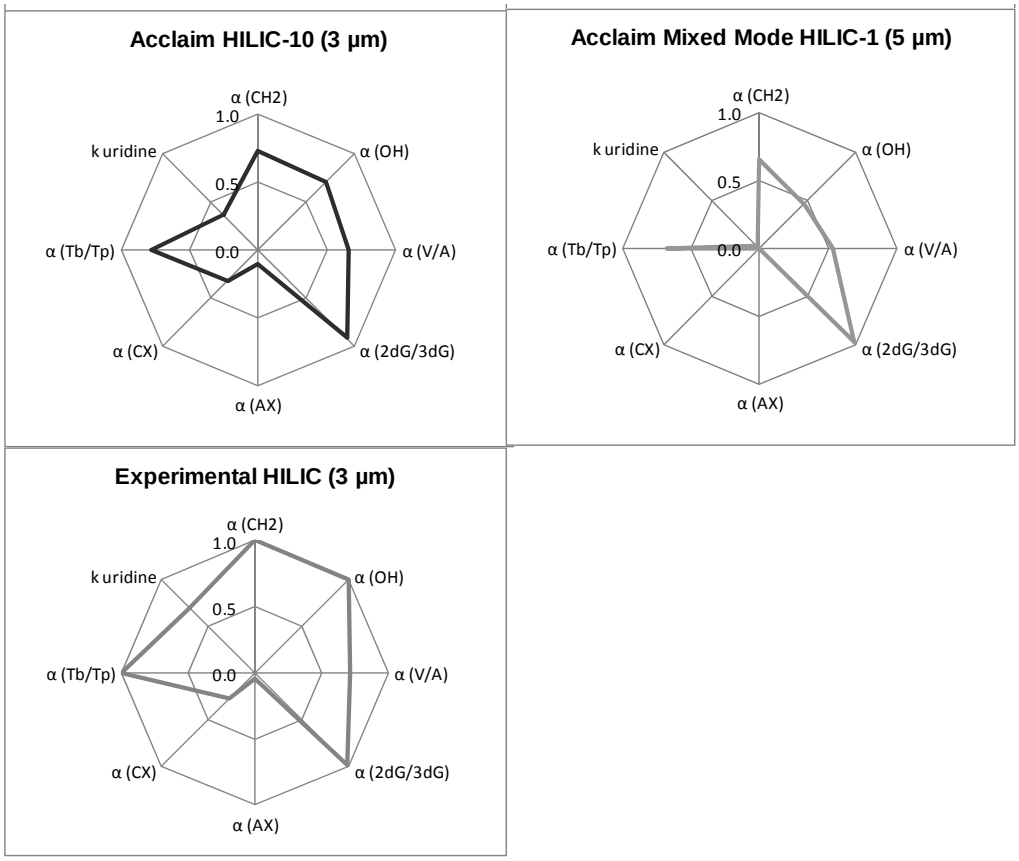


Figure 7. Radar plots for HILIC stationary phases (data were normalized to 1 as the greatest value).

Table 6. Separation factors for α (Tb/Tp).

Column name	**α (Tb/Tp)**	**pH conditions of stationary phase**
Syncronis HILIC (5 µm)	1.000	Neutral
Hypersil GOLD HILIC (5 µm)	1.000	
Acclaim HILIC-10 (3 µm)	1.000	
Hypercarb (5 µm)	0.737	Basic
Acclaim Mixed Mode HILIC-1 (5 µm)	0.860	
Acclaim Trinity P1 (3 µm)	0.671	
Syncronis Silica (5 µm)	1.151	Acidic
Hypersil GOLD Silica (1.9 µm)	1.102	
Hypersil GOLD Silica (5 µm)	1.091	
Accucore HILIC (2.6 µm)	1.189	
Experimental HILIC (3 µm)	1.269	

From the radar plots illustrated in Figure 7 it is interesting to observe that α (CH2) and α (OH) show a positive correlation for all the materials. A similar correlation between α (CH2) and α (OH) was observed by Tanaka and his group [31]. A tentative interpretation for this observation is that the chemistry of the stationary phases does not have a substantial role on the selectivity of these two groups. On the other hand, $k_{uridine}$ data demonstrate that the stationary phase chemistry has an effect on the absolute retention, probably due to the absolute volume of the water layer. It can be seen that the bare silica materials, the Trinity P1 and the mixed mode HILIC-1 exhibit lower values for $k_{uridine}$. Syncronis HILIC and PGC demonstrated to be the most retentive materials, showing the largest retention for uridine. The bare silica of Hypersil GOLD provided different $k_{uridine}$, α (OH) and α (CH2) values from the silica in Accucore HILIC and Syncronis Silica. These differences could be due to differences in pore volume, surface area and particle morphology for the three silica types. Syncronis Silica showed a higher retentivity than Hypersil GOLD Silica due to its higher nominal surface area. Accucore HILIC, in turn demonstrated higher $k_{uridine}$, α (OH) and α (CH2) values than the other bare silica columns. This is likely to be due to the higher surface area per column within Accucore columns. Although Accucore has a lower nominal surface area (in terms of m^2/g), because it is a superficially porous material, when packed into a column it has higher g/column than a fully porous material. As a result, within an Accucore column, overall there is more surface available for interaction.

PGC showed the lowest values for α (OH) and α (CH2).

From Table 4 it can be observed that Syncronis HILIC provided the best selectivity for α (V/A) and α (2dG/3dG). Similar data were reported by Tanaka's group for Nucleodur HILIC and ZIC-HILIC [31]. Mixed Mode HILIC-1 cannot discriminate between the two configurational isomers, as demonstrated by the α (V/A) value of 1.0. This diol material showed a similar α (2dG/3dG) value to the 1.06 value reported by Tanaka *et al.* for Lichrosphere Diol [31]. Hypercarb showed the highest α (V/A) amongst the columns evaluated, indicating that PGC provides the best separation for a mixture of nucleosides. This is in agreement with the high stereoselectivity of PGC [33].

The fact that α (2dG/3dG) values are about 1.1 for most materials (apart from Hypercarb and HILIC-10) would indicate less specificity for positional isomers. From the radar plots it can be observed some correlation between α (V/A) and α (2dG/3dG) for most phases, apart from PGC, although the small variations for α (2dG/3dG) data are not sufficiently significant. These small variations were also observed on the materials characterized by Tanaka and his group [31], suggesting that these probes are not selective enough. From Table 5 it can be observed that Hypersil GOLD HILIC and Acclaim Trinity P1 have the strongest anion interactions; these results are expected, considering that both materials posses amino groups, which work as AX functionalities at the pH experimental conditions of 4.7. The bare silica materials exhibited the highest α (CX) values; bare silica phases are known to possess cation exchange ability due to their silanols (SiOH) functionality.

For the mixed mode HILIC-1 the value for α (AX) was not reported, and the value for α (CX) was zero, since SPTS eluted faster than t_0 and TMPAC co-eluted with t_0. PGC also demonstrated α (CX)= 0. It has been observed that some ligands exclude TMPAC and SPTS from the pore volume, resulting in these compounds not being retained [31]. Pore exclusion could be advocated for the early elution of SPTS and TMPAC experienced on the mixed mode HILIC-1.

The lack of retention observed for TMPAC on PGC is in agreement with Elfakir *et al.*, who demonstrated strong retention capabilities for anionic species and weaker retentions for cationic species on Hypercarb [36].

From the AX and CX characterization study it can be concluded that cation exchange interactions have important effects in HILIC on bare silica phases. Syncronis HILIC showed considerable CX character, due to the sulfo group in the phase; however, the α (CX) value for Syncronis HILIC was much lower than the values recorded by Tanaka's group for Nucleodur HILIC and

ZIC-HILIC (3.46 and 4.41 respectively) [31]. Experimental HILIC also demonstrated some CX character. The degree of ion exchange interactions has a major impact on the shape of the radar plots, as illustrated in Figure 7, with a distinct dichotomy between (i) the bare silica materials, which have strong cation exchange ability, and (ii) Trinity P1 and GOLD HILIC, which exhibit strong anion exchange activity. Very little ion exchange interactions were demonstrated by PGC, HILIC-10 and mixed mode HILIC-1.

The investigation into the pH on the stationary phase surface lead to the following classification: (i) α (Tb/Tp) = 1.0, which indicates that theophylline and theobromine are not separated; (ii) α (Tb/Tp) > 1, which illustrates that theobromine is more strongly retained than theophylline, probably because it can orient itself with the nitrogen in position 1 more in proximity to these stationary phases (whereas theophylline, with a methylene group on the nitrogen in position 1 is more sterically hindered); (iii) α (Tb/Tp) < 1, which denotes that theophylline is more strongly retained than theobromine; its molecule orientation must influence the access to these stationary phase surfaces. In the study by Lämmerhofer *et al.* [28] it was shown that basic stationary phases give α (Tb/Tp) < 1; neutral phases give α (Tb/Tp) = 1 and acidic phases give α (Tb/Tp) > 1. Based on these observations, the materials under current investigation were classified accordingly, as reported in Table 6. The acidic phases comprise the silica and the amide materials. Amide materials are supposedly neutral in terms of the nature of their functionality [31], but experimental HILIC demonstrated a high α (Tb/Tp) value and it could therefore be expected to show an acidic nature in terms of retentions. The zwitterionic material, Syncronis HILIC proved to be neutral. Interestingly, Tanaka and his group found that some zwitterionic phases (i.e. ZIC-HILIC) were acidic, whereas others (i.e. Nucleodur HILIC) were neutral [31]. Irgum *et al.* confirmed these findings and suggested that ligand loading could be responsible for this dual nature of zwitterionic materials, since ZIC-HILIC columns are polymerically functionalized, whereas Nucleodur HILIC columns are monomerically functionalized and therefore have a lower ligand loading [32]. Syncronis HILIC, being monomerically functionalized and neutral, confirms Irgum's suggestion.

CONCLUSIONS

The investigation carried out in this study lead to the characterization of the HILIC stationary phases in terms of:

- selectivity based on a methylene group;
- hydrophilic selectivity based on an hydroxy group;
- regio isomer selectivity;
- configurational isomer selectivity;
- ion-exchange properties;
- acidic-basic nature of the stationary phases.

The findings for this study were summarized as radar graphs, which exhibited several patterns of data sets. However, two prevailing selectivity trends could be identified: ion-exchange interactions and hydrophilic partitioning. These trends had a significant influence on the shapes of the radar graphs, and allowed to separate the HILIC stationary phases in two groups:

1. Phases containing amides and zwitterionic groups; they demonstrated higher hydrophilic retention, better selectivity for the test compounds and little ion exchange interactions. These materials demonstrated suitability for a wide range of analytes; in particular, they should be recommended when analyzing acids, bases and compounds that do not have ion exchange functionalities.
2. Phases containing hydroxy and amino groups and bare silica materials; they showed relatively low retention and selectivity, but considerable ion exchange activity. These materials should be avoided when analytes are acidic or basic, to minimize secondary ion exchange interactions, which in turn would lead to peak tailing and lower efficiency.

This classification is coherent with the categories proposed by Tanaka *et al.* [31] and with the clusters highlighted by Principal Component Analysis carried out by Irgum and his group [32].

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