Technical Note 20741

Chromatographic Characterization of Stationary Phases for Hydrophilic Interaction Liquid Chromatography

Monica Dolci, Thermo Fisher Scientific, Runcorn, Cheshire, UK

Key Words

Hydrophilic interaction chromatography, HILIC, chromatographic characterization, structural selectivity, ion exchange interactions

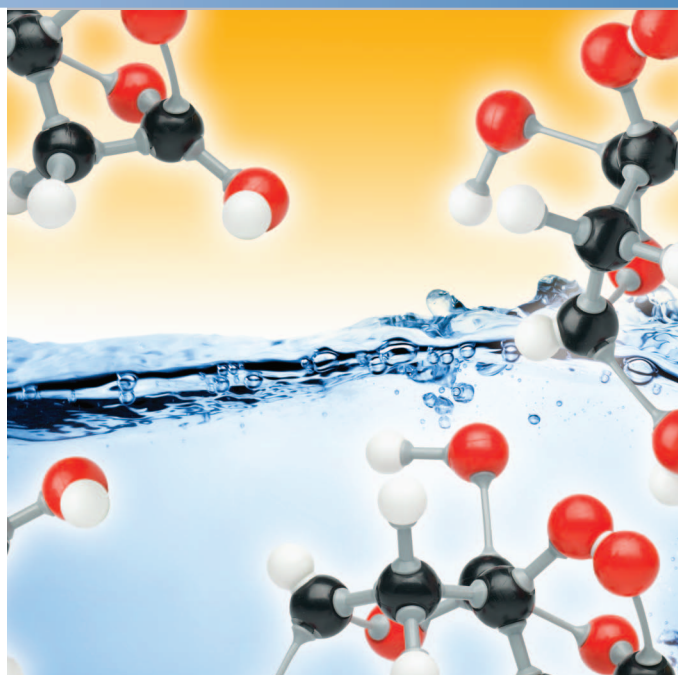
Abstract

The work presented herein summarizes the results of a chromatographic characterization study of HILIC stationary phases involving ten silica-based columns, including unmodified silica, amino, diol, anion exchanger, and zwitterionic materials, and a porous graphitic carbon (PGC) column. The column characterization methodology allowed the identification and understanding of primary and secondary retention mechanisms and the classification of the HILIC stationary phases according to their chromatographic properties. This ultimately can be used as a column selection tool during method development in HILIC separations.

Introduction

Hydrophilic interaction chromatography (HILIC) can be described as a reversed reversed-phase chromatography performed using a polar stationary phase (for example, unmodified silica, amino, or diol bonded phases). 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 retention mechanisms in HILIC are complex but are believed to be a combination of hydrophilic partitioning interaction and secondary electrostatic and hydrogen bonding interactions. These mechanisms result in an elution order that is roughly the opposite of that in reversed phase [1]. Although 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 important in matching the column chemistry to the analyte functional groups. In addition to retention characteristics and selectivity, separation efficiency is the key parameter that can be critical for a specific separation [3]. It was therefore necessary to characterize Thermo Scientific™ HILIC phases to highlight these cardinal aspects of method development.



The objectives of this study were:

- Perform hydrophilicity and hydrophobicity comparison of the columns in the study.
- Carry out HILIC characterization testing that probes specific secondary interactions according to Tanaka HILIC characterization testing regime [3].
- Classify the HILIC materials in the study on the basis of their chromatographic properties.
- Provide a tool to facilitate column selection for target separations.

Thermo SCIENTIFIC

Column Name	Phase Type	Column Dimension (mm)	Surface Area (m^2/g)	Pore Size (Å)
Syncronis HILIC (5 µm)	Zwitterion	100 × 4.6	320	100
Hypersil GOLD HILIC (5 µm)	Polyethyleneimine	100 × 4.6	220	175
Hypersil GOLD Silica (5 µm)	Unbonded Silica	100 × 4.6	220	175
Hypersil GOLD Silica (1.9 µm)	Unbonded Silica	100 × 2.1	220	175
Syncronis Silica (5 µm)	Unbonded Silica	100 × 4.6	320	100
Accucore HILIC (2.6 µm)	Unbonded Silica	100 × 4.6	130	80
Acclaim Mixed Mode HILIC-1 (5 µm)	Mixed Mode Diol	150 × 4.6	300	120
Acclaim HILIC-10 (3 µm)	Proprietary	150 × 4.6	300	120
Acclaim Trinity P1 (3 µm)	NSH*	150 × 3.0	100	300
Experimental HILIC (3 µm)	Polyacrylamide	150 × 3.0	220	90
Hypercarb (5 µm)	PGC	100 × 4.6	120	250

*Nanopolymer silica hybrid

Table 1: Specifications of the HILIC stationary phases characterized

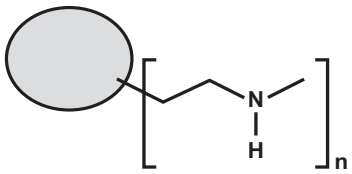
The stationary phases investigated in this study are summarized in Table 1.

- The Thermo Scientific™ Syncronis™ HILIC column contains a zwitterionic stationary phase, comprising sulfonic acid and quaternary amine groups, that provides weak electrostatic interactions. The charge density of this material is pH-independent, given the presence of two functional groups of opposite charge.
- The Thermo Scientific™ Hypersil GOLD™ HILIC stationary phase has a weak anion exchanger, based on a polymeric amine ligand, polyethyleneimine. The main benefit of using a charged stationary phase lies in the extra selectivity brought about by the possible electrostatic interactions with the analyte. For Hypersil GOLD HILIC columns, the strength of these interactions depends on the ionization of the solute and the stationary phase (the charge density is therefore pH-dependent). High buffer concentrations may be necessary in order to disrupt these interactions and allow the analyte to elute.
- Hypersil GOLD Silica, Thermo Scientific™ Accucore™ HILIC, and Syncronis Silica columns contain unmodified silica, with different pore size, surface area, particle size characteristics, and particle morphology, as detailed in Table 1.
- The Thermo Scientific™ Hypercarb™ column (Porous Graphitic Carbon, PGC) contains fully porous particles made up of graphitic layers of hexagonally arranged carbon atoms, with no functional groups on the surface. The surface of PGC is not hydrophilic, but can be used to retain polar compounds in both typical reversed phase and HILIC mobile phase conditions [4].
- The Thermo Scientific™ Acclaim™ HILIC-10 column's stationary phase is based on silica covalently modified with an hydrophilic group.
- The Acclaim Mixed Mode HILIC-1 column's stationary phase consists of a hydrophobic alkyl chain with a terminal diol group.
- The experimental HILIC stationary phase contains a polyacrylamide functionality.
- The Thermo Scientific™ Acclaim™ Trinity™ P1 column is based on Nanopolymer Silica Hybrid (NSH) technology and consists of high purity silica particles coated with charged nanopolymer beads. This unique surface chemistry provides reversed phase, anion exchange (tertiary amine), and cation exchange (fully sulfonated polymer beads electrostatically attached to the outer surface of the bonded silica) properties.

Some of the column chemistries are illustrated in Figure 1.

Considering the variations in stationary phases, a HILIC test scheme was adopted to evaluate primary and secondary interactions that can lead to changes in selectivity for partial structural differences. The data from this characterization testing were used to classify Thermo Scientific HILIC stationary phases on the basis of their properties.

a)



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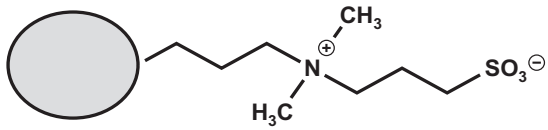

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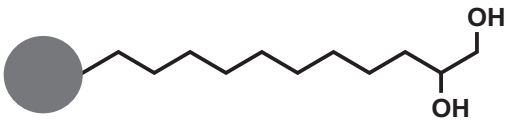

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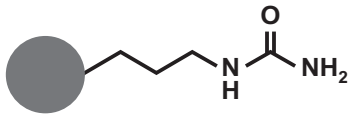
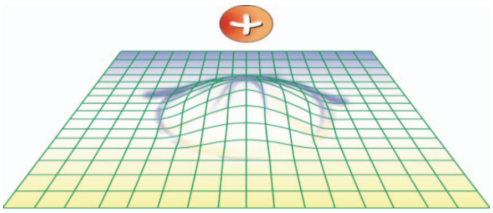
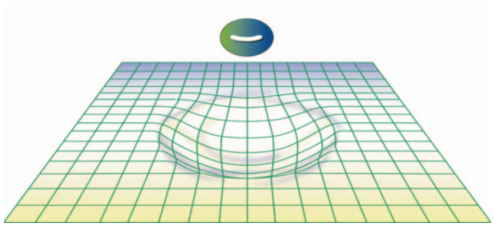


Analyte with electron-withdrawing properties approaching the graphite surface

Analyte with electron-donating properties approaching the graphite surface





e)

Figure 1: Schematic representation of the chemistries for: a) Hypersil GOLD HILIC; b) Syncronis HILIC; c) Acclaim Mixed Mode HILIC-1; d) Acclaim HILIC-10; e) Schematic representation of charge induced interaction on the PGC surface

Experimental

Separation Conditions	
Instrumentation:	HPLC system equipped with a quaternary pump, a DAD detector, a degasser, a column heater, and an autosampler.
Columns:	Listed in Table 1.
Mobile phase:	For test mixtures 1–7: Acetonitrile / ammonium acetate pH 4.7 (90:10 v/v) (20 mM on the column)
	For test mixture 8: Acetonitrile / ammonium acetate pH 5.2 (various ratios) (10 mM on the column)

Instrument Setup
For test mixtures 1–7: Flow rate: 0.5 mL/min; UV: 254 nm; Injection volume: 5 µL; Column temperature: 30 °C.
For test mixture 8: Flow rate: 1.0 mL/min; UV: 254 nm; Injection volume: 5 µL; Column temperature: 30 °C.

Sample Preparation
Individual compounds, their structures, and physiochemical properties are given in Table 2. 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: 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).
Acetone was used as t_0 marker (instead of toluene) on the Hypercarb column.
Six replicate injections were performed on each column. Retention times, retention factor, selectivity, peak area, and peak asymmetry values were recorded (reported in the Appendix).

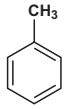
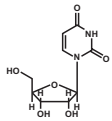
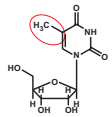
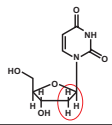
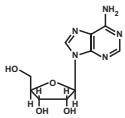
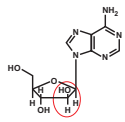
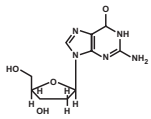
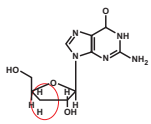
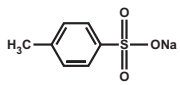
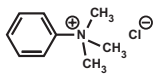
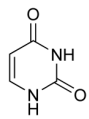
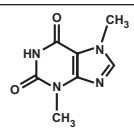
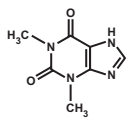
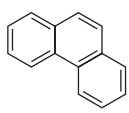
Chromatographic Probes	Molecular Structure	Variable	pKa	LogD	Test Mixture
Toulene		t_0 marker	41	2.72	all
Uridine		Hydrophobic/ hydrophilic interaction	12.6	-1.58	1, 2
5-Methyluridine		Hydrophobic interaction	12.0	-1.02	1
2'-Deoxyuridine		Hydrophilic interaction	13.9	-1.26	2
Adenosine		Configurational isomers selectivity	13.9	-1.03	3
Vidarabine		Configurational isomers selectivity	13.9	-1.02	3
2'-Deoxyguanosine		Regio isomers selectivity	13.5	-1.14	4
3'-Deoxyguanosine		Regio isomers selectivity	13.5	-1.14	4
Sodium p-toluenesulfonate		Anion exchange selectivity	-2.8	0.88	5
N,N,N-trimethylphenylammonium chloride		Cation exchange selectivity		-2.31	6
Uracil		Hydrophilic interaction	13.8	-1.08	5, 6, 8
Theobromine		Acidic-basic nature of stationary phase	10	-1.06	7
Theophylline		Acidic-basic nature of stationary phase	8.6	-2.51	7
Phenanthrene		Hydophobic interaction		4.55	8

Table 2: List of chromatographic probes, their physiochemical properties and nature of interactions tested

Hydrophobic and Hydrophilic Interactions: Separation Factors Provided by a Methylene Group, α (CH_2), and a Hydroxy Group, α (OH)

The degree of surface coverage of silica by hydrophobic groups is a useful parameter in both reversed phase LC 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 (CH_2). In this study α (CH_2) was obtained from a comparison of the retention factor for uridine, k (uridine), and the retention factor for 5-methyluridine, k (5-methyluridine). Figure 2 shows chromatograms obtained for this test mixture 1.

From Figure 2 it can be seen that apart from the Hypercarb and Acclaim HILIC-1 columns, uridine is more retained than 5-methyluridine (5MU), which reflects the fact that uridine is more hydrophilic than 5MU. With the Hypercarb column, the more hydrophilic uridine elutes first. On the Acclaim HILIC-1 column, uridine and 5MU are not resolved.

Average α (CH_2) values were obtained from the average ratio of k (uridine) and k (5-methyluridine) for each phase and are summarized in Table 3. Examples of individual values and mean values for two representative tests on two columns are given in the Appendix.

The degree of hydrophilic interaction between the stationary phase and the test compounds was assessed using the selectivity for an hydroxy group, α (OH). Test mixture 2 was run on each column, with the resulting chromatograms shown in Figure 3. In this study, α (OH) was obtained from a comparison of k (uridine) and k (2'-deoxyuridine). The resulting α (OH) values for the stationary phases tested are reported in Table 3.

From Figure 3 it can be seen that apart from the Hypercarb and Acclaim HILIC-1 columns, uridine (U) is more retained than 2'-deoxyuridine (2dU); this reflects the fact that U is more hydrophilic than 2dU. The Hypercarb and Acclaim HILIC-1 columns can not discriminate between U and 2dU under the test conditions used in this study.

From Table 3 it can be seen that the Syncronis HILIC, Accucore HILIC, and experimental HILIC columns exhibited the greater selectivity for α(CH_2) and α(OH). Amongst the stationary phases studied, Syncronis HILIC and Hypercarb demonstrated to be the most retentive materials, showing the largest retention for uridine. The bare silica of Hypersil GOLD Silica provided different kU, α(OH) and α(CH_2) values from the silica in Accucore HILIC and Syncronis Silica. These differences could be due to differences in pore volume and surface area for the three silica types. Syncronis Silica showed a higher retentivity than Hypersil GOLD Silica due to its higher surface area. The solid core, silica-based Accucore HILIC column, in turn demonstrated higher kU, α(OH) and α(CH_2) values than the other bare silica columns, possibly due to its smaller pore volume.

The lowest values for α (OH) and α (CH_2) (lowest α values being equal to 1) were demonstrated by Acclaim Mixed Mode HILIC-1. Hypercarb showed a value of 1 for α (OH), and proved to be the second most hydrophobically selective material, since its α (CH_2) value is farther from 1 than most of the other phases α (CH_2) data.

Column Name	α (CH_2)	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

Table 3: Separation factors for methylene α (CH_2) and hydroxy α (OH) groups and retention factor for uridine

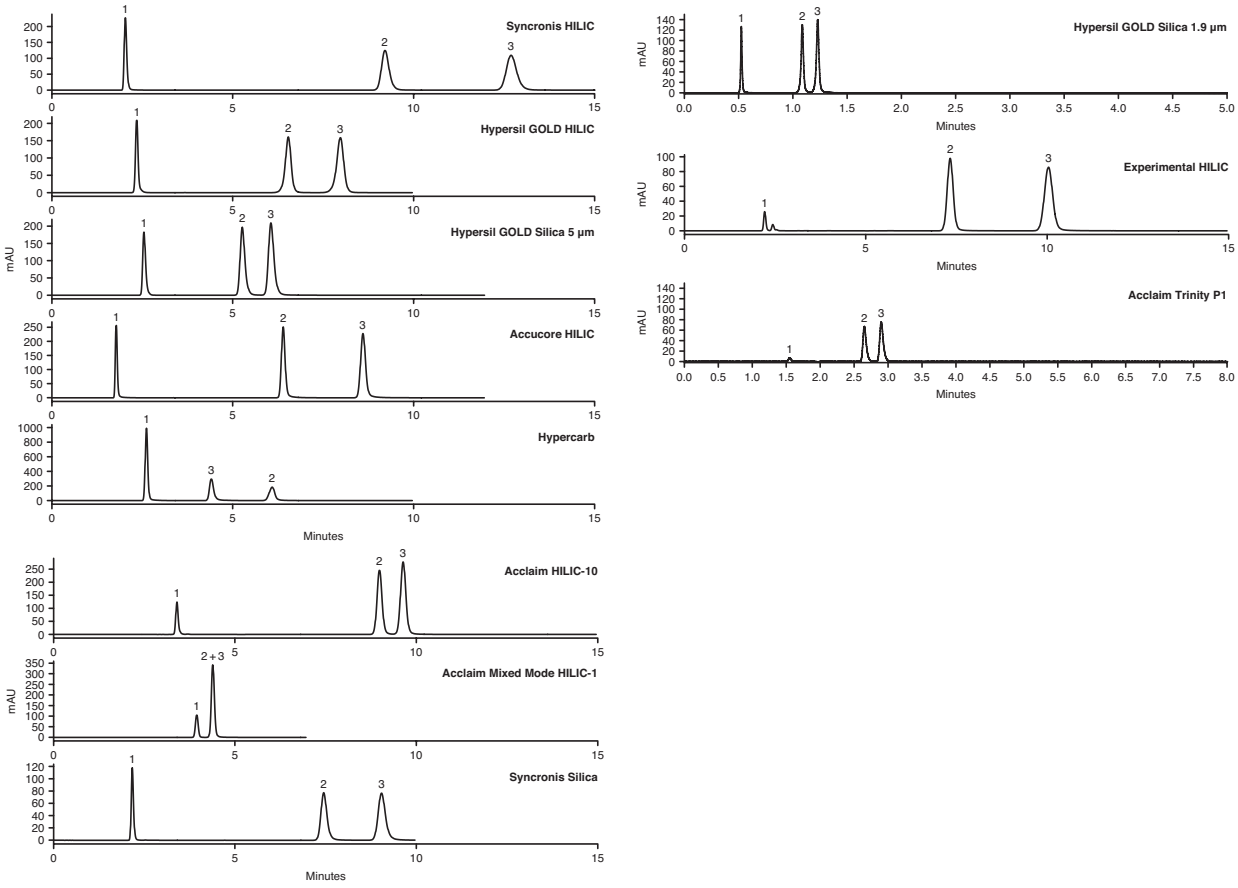


Figure 2: Chromatograms for α (CH_2) test. Analyte: 1. toluene; 2. 5-methyluridine; 3. uridine

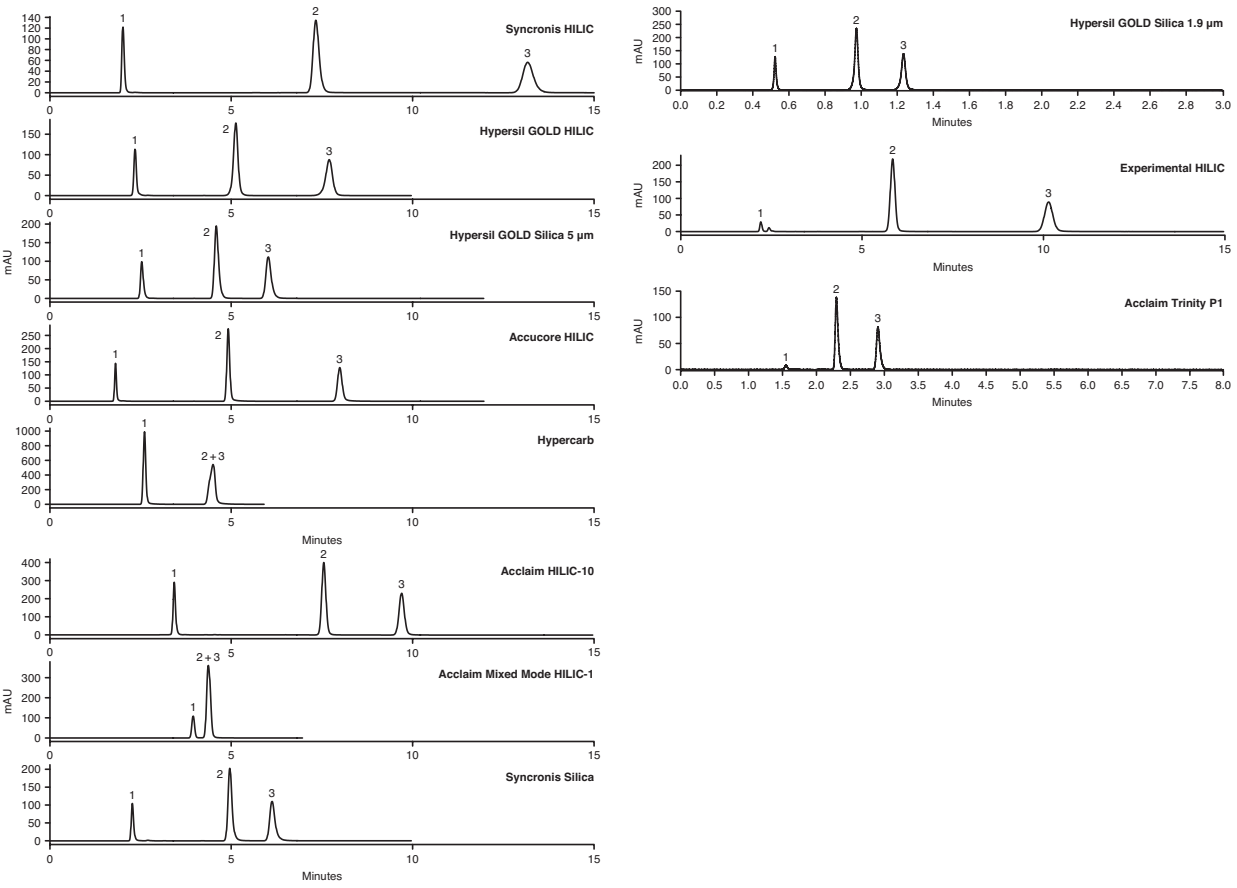


Figure 3: Chromatograms for α (OH) test. Analyte: 1. toluene; 2. 2'-deoxyuridine; 3. uridine

Isomeric Selectivity: Separation Factors Provided by Configurational Isomers α (V/A) and Regio Isomers, α (2dG/3dG)

Test mixtures 3 and 4 (which contain configurational and regio isomers, respectively) were used in this study. The resulting chromatograms are shown in Figure 4 and Figure 5. In this study, α (V/A) was obtained from a comparison of k (vidarabine) and k (adenosine). α (2dG/3dG) was calculated from the k (2' deoxyguanosine)/k (3' deoxyguanosine) ratio. The resulting mean α (V/A) and α (2dG/3dG) values for each of the stationary phases tested are reported in Table 4.

The configurational isomers co-elute on the Acclaim Mixed Mode HILIC-1 column, but are separated by all the other 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 achieved on the Acclaim Trinity P1, Hypersil GOLD Silica, Hypersil GOLD HILIC, Hypercarb, Acclaim HILIC-1 and Acclaim HILIC-10 columns. With the exception of Acclaim HILIC-10, 2'-deoxyguanosine is more retained than 3'-deoxyguanosine on all of the columns.

The Syncronis HILIC column provided good selectivity for α (2dG/3dG). Similar data were reported by Tanaka's group for Nucleodur® HILIC and ZIC® HILIC colums [3]. The Acclaim Mixed Mode HILIC-1 column cannot discriminate between the two configurational isomers. This diol material showed similar α (2dG/3dG) data to what Tanaka reported for the LiChrosphere® Diol column [3]. From Table 4 it can be concluded that the configurational isomer selectivity data have more variation than the regio isomer selectivity data. The small variations for α (2dG/3dG) were also observed on the materials tested by Tanaka and his group. The Hypercarb stationary material showed the highest α (V/A) amongst the columns evaluated, indicating that it provides the best separation for these configurational isomers. This is in agreement with the high stereoselectivity of PGC [4].

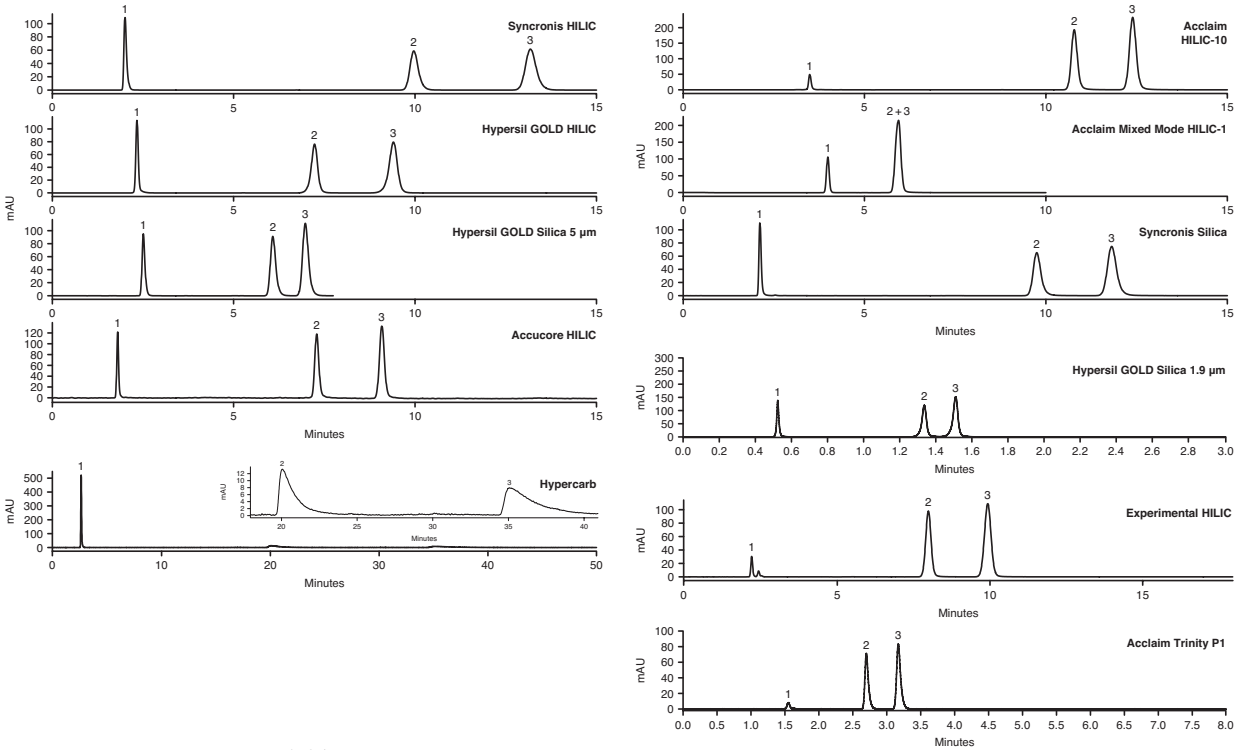


Figure 4: Chromatograms for α (V/A) test. Analyte: 1. toluene; 2. adenosine; 3. vidarabine

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

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

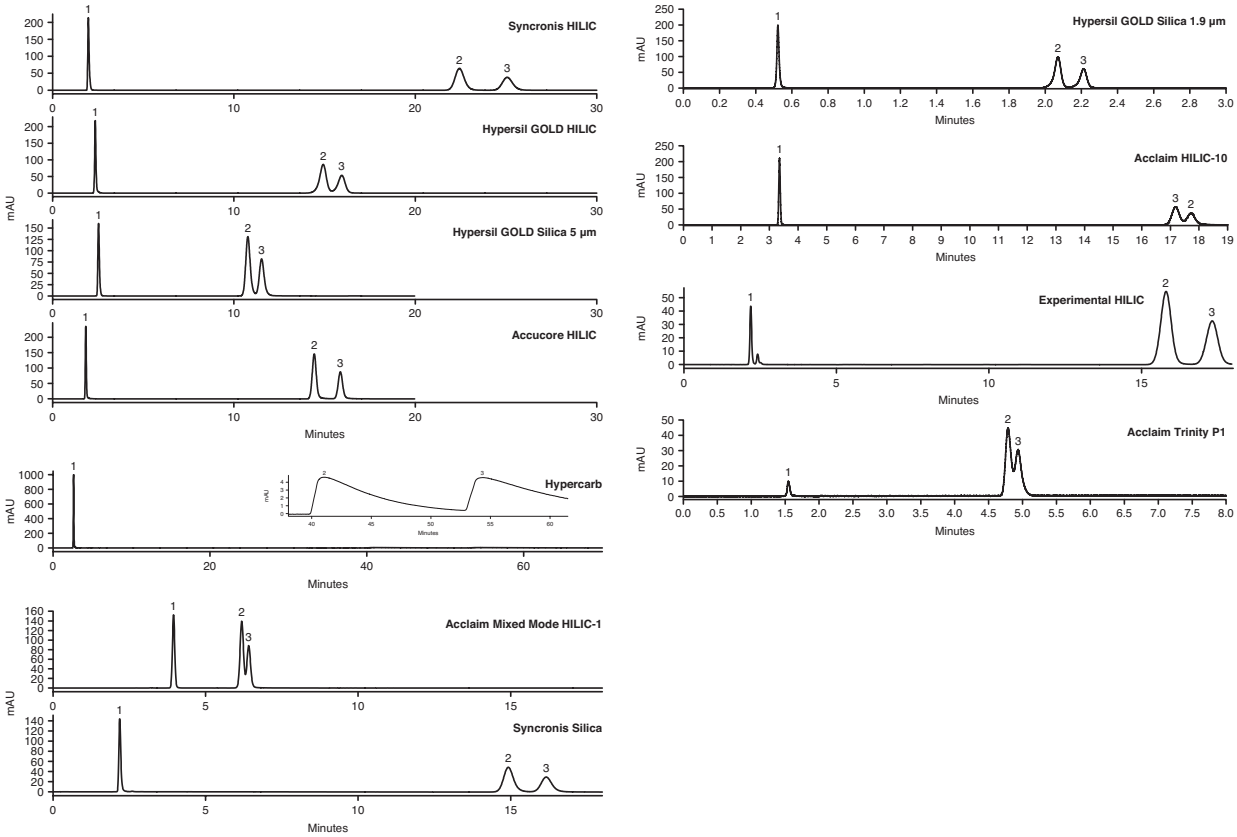


Figure 5: Chromatograms for α (2dG/3dG) test. Analyte: 1. toluene; 2. 3'-deoxyguanosine; 3. 2'-deoxyguanosine

Anion and Cation Exchange Interactions, α (AX) and α (CX)

Ion-exchange interactions can be influential in HILIC, particularly when separating ionic species, since they can lead to drastic changes in selectivity. To estimate the degree of ion exchange capability of the stationary phases, a relatively hydrophobic organic anion, sodium p-toluenesulfonate (SPTS, Test mixture 5), and a relatively hydrophobic organic cation, N,N,N-trimethylphenyl-ammoniumchloride (TMPAC, Test mixture 6), were chosen. It is reasonable to postulate that these compounds would also be retained by hydrophilic interactions, 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 is shown in Figure 6 and Figure 7, respectively. The resulting mean separation factors, α (AX) and α (CX) for the stationary phases tested are reported in Table 5.

Figure 6 shows that for some materials SPTS elutes before uracil, the exceptions being:

- Hypersil GOLD HILIC and Acclaim Trinity P1 columns, where SPTS elutes after uracil
- Acclaim Mixed Mode HILIC-1 column, where SPTS is not retained and it elutes before toluene
- Acclaim HILIC-10 column, where SPTS co-elutes with uracil

From Figure 7 it can be seen that TMPAC elutes after uracil, apart from:

- Hypercarb column, where it is not retained, eluting before acetone (t_0 marker for Hypercarb)
- Hypersil GOLD HILIC column, where it elutes in front of uracil
- Acclaim Mixed Mode HILIC-1 column, where it co-elutes with toluene
- Acclaim Trinity P1 column, where it co-elutes with uracil

From Table 5 it can be concluded that Hypersil GOLD HILIC and Acclaim Trinity P1 phases 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 silanol (SiOH) functionality. The pKa of silanols is around 4.7, thus 50% of them exist as SiO- groups under the pH conditions used in this study (pH = 4.7). From this 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 presence of the sulfo group. It must also be highlighted that Acclaim HILIC-10 and Acclaim Mixed Mode HILIC-1 have some anionic- and cationic-exchange properties, respectively. However, under the current experimental conditions these ionic properties are not demonstrated.

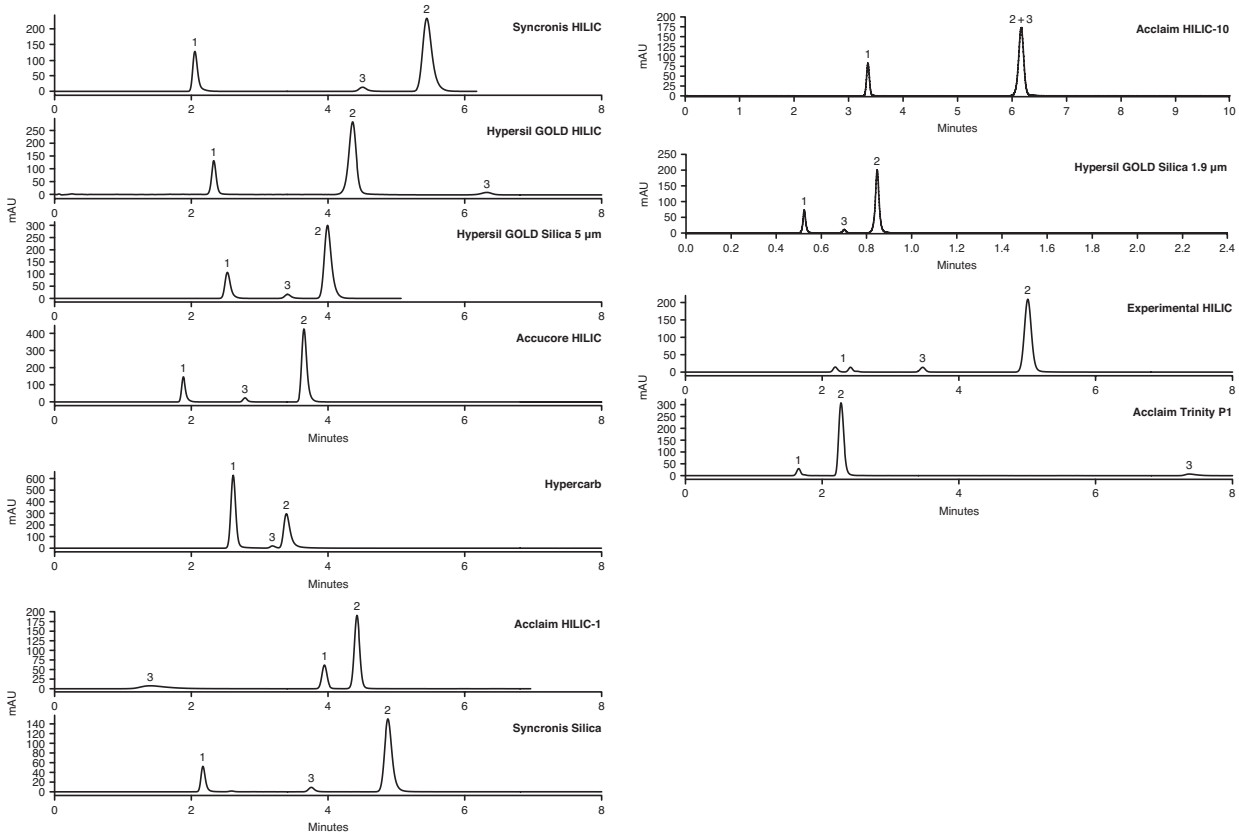


Figure 6: Chromatograms for α (AX) test. Analyte: 1. toluene; 2. uracil; 3. sodium p-toleuenesulfonate, SPTS

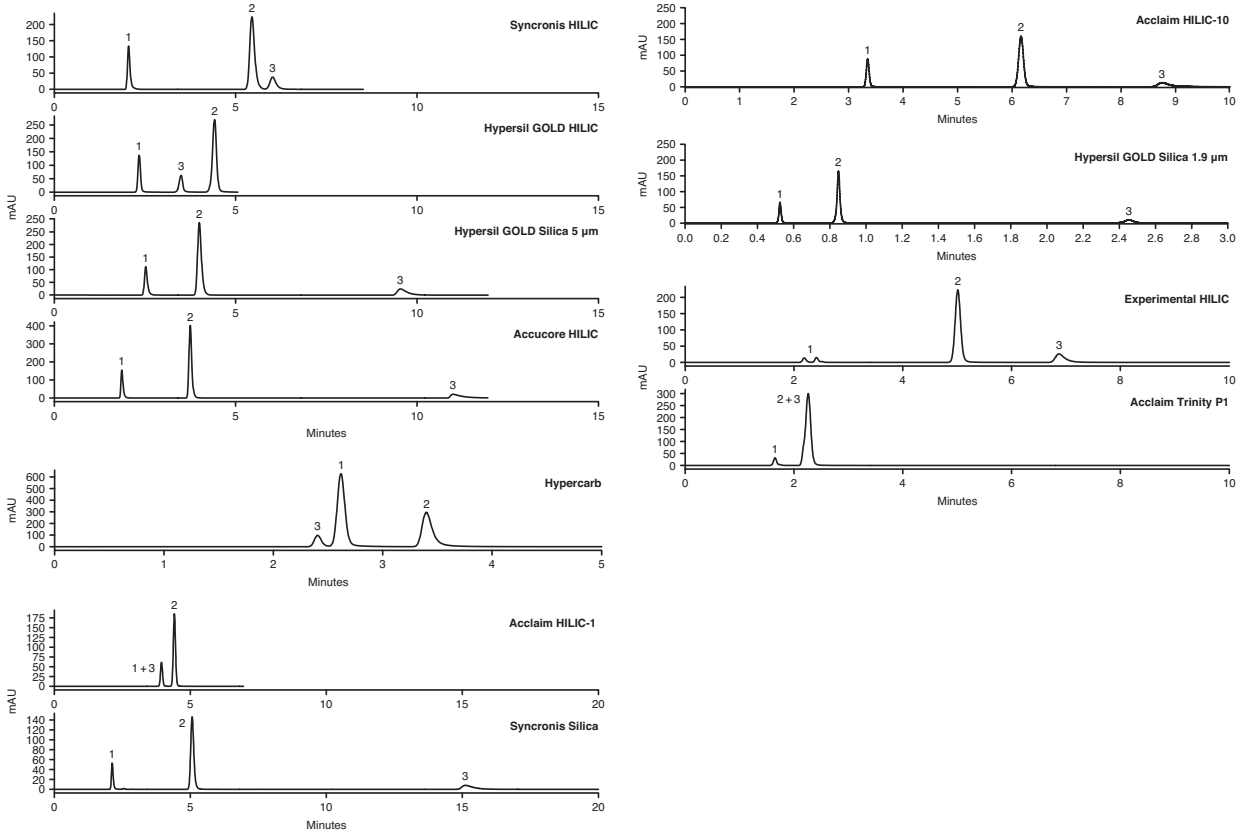


Figure 7: Chromatograms for α (CX) test. Analyte: 1. toluene; 2. uracil; 3. N,N,N-trimethylphenylammoniumchloride, TMPAC

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	–

Table 5: Separation factors for anion exchange interactions α (AX) and cation exchange interactions α (CX)

Evaluation of the Acidic-Basic Nature of the Stationary Phase Surface, α (Tb/Tp)

Many compounds analyzed in HILIC have ionizable functional groups. Knowing the acid-base properties of the stationary phase is important for controlling the separation. Test mixture 7 was used for this investigation. Chromatograms are given in Figure 8. k (theobromine)/k (theophylline), k (Tb)/k (Tp) values are reported in Table 6. 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.

As shown in Figure 8, theophylline and theobromine are not separated on Syncronis HILIC, Hypersil GOLD HILIC, Hypersil GOLD Silica 5 µm and Acclaim HILIC-10 columns. On Accucore HILIC, Syncronis Silica, Hypersil GOLD Silica 1.9 µm, and Experimental HILIC columns, theobromine is more strongly retained than theophylline. On Hypercarb, Acclaim Mixed Mode HILIC-1, and Acclaim Trinity P1 columns, theophylline is more strongly retained than theobromine.

In the study by Lämmerhofer et al. [5] 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, 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 [3], 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 in the Syncronis HILIC column 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 [3]. 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 [6]. Syncronis HILIC columns, being monomerically functionalized and neutral, confirm Irgum's suggestion.

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	
Acclaim Mixed Mode HILIC-1 (5 µm)	0.860	Basic
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	

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

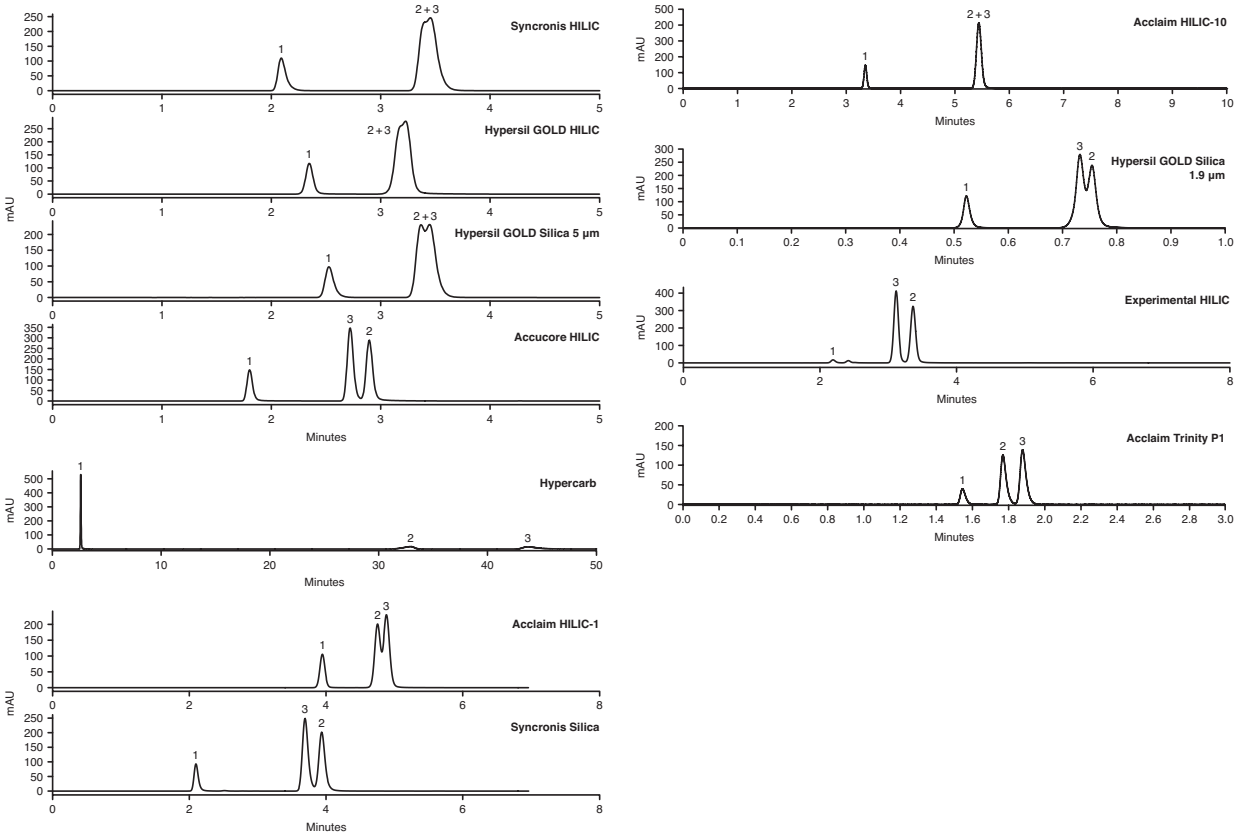


Figure 8: Chromatograms for α (Tb/Tp) test. Analyte: 1. toluene; 2. theobromine; 3. theophylline

Comparison of Overall Selectivity: Radar Plots of the Stationary Phases

The results generated from the eight characterization tests were plotted in radar plots, so that the characteristics of each phase can be visually assessed and easily compared. The resulting radar plots, in which each axis represents one of the parameters measured, are shown in Figure 9.

From the radar plots and from Figure 10, it is interesting to observe that α (CH_2) and α (OH) show a positive correlation for all the materials. A similar correlation between α (CH_2) and α (OH) was observed by Tanaka and his group [3]. 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 columns exhibit lower values for k (uridine). Syncronis HILIC and PGC columns demonstrated to be the most retentive materials. The bare silica of the Hypersil GOLD column provided different k (uridine), α (OH) and α (CH_2) values from the silica in the Accucore HILIC and Syncronis Silica columns. These differences could be due to differences in pore volume, surface area, and particle morphology for the three silica types. The Syncronis Silica column showed a higher retentivity than the Hypersil GOLD Silica column due to its higher nominal surface area. The Accucore HILIC column, in turn demonstrated higher k (uridine), α (OH), and α (CH_2) values than the other bare silica columns. This is likely due to the higher surface area per column within Accucore columns. Although the Accucore material has a lower nominal surface area (in terms of m^2/g), because it is a solid core 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).

The fact that α (2dG/3dG) values are about 1.1 for most materials (apart from Hypercarb and Acclaim HILIC-10 materials) 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 [3], suggesting that these probes are not selective enough.

For Acclaim Mixed Mode HILIC-1 material, the value for α (AX) was not reported, and the value for α (CX) was zero because SPTS eluted faster than t_0 and TMPAC co-eluted with t_0. PGC material 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 [3]. Pore exclusion could be advocated for the early elution of SPTS and TMPAC observed 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 columns [7].

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 material showed considerable CX character, due to the sulfo group in the phase; however, the α (CX) value was much lower than the values recorded by Tanaka's group for Nucleodur HILIC and ZIC-HILIC material (3.46 and 4.41 respectively) [3]. Experimental HILIC also demonstrated some CX character. The degree of ion exchange interactions has a major impact on the shape of the radar plots, with a distinct dichotomy between (i) the bare silica materials, which have strong cation exchange ability, and (ii) Trinity P1 and Hypersil GOLD HILIC materials, which exhibit strong anion exchange activity. Very little ion exchange interactions were demonstrated by PGC, HILIC-10 and mixed mode HILIC-1 materials.

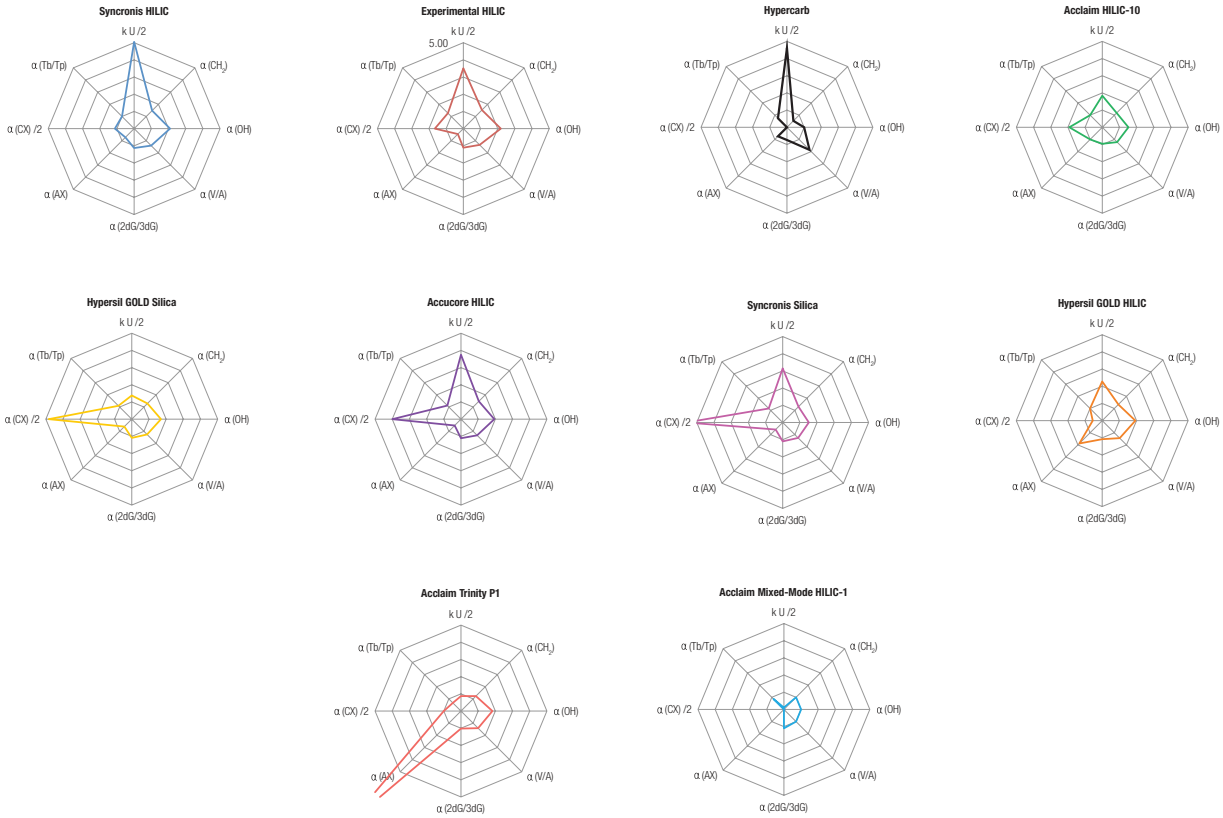


Figure 9: Radar plots for HILIC stationary phases

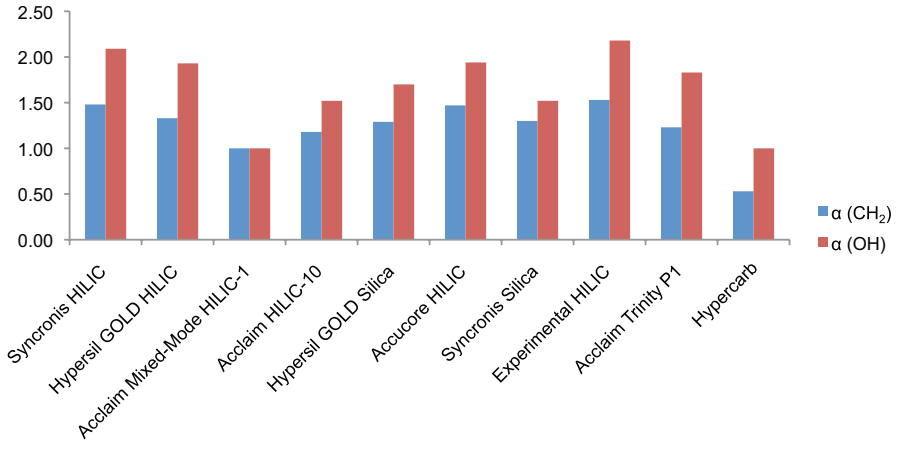


Figure 10: α (CH_2) and α (OH) correlation

Organic solvent effect

In this study the retention behavior dependency on organic solvent concentration was investigated. This work was based on the research carried out by Liu and Pohl [8] on Acclaim Trinity P1 columns. Phenanthrene (t_0 marker) and uracil were used as test probes for hydrophobic and hydrophilic interactions, respectively. A series of mobile phases was prepared by proportioning the acetonitrile percentage (between 5% and 95%), while ammonium acetate buffer was kept constant at 10 mM, pH 5.2. The retention factor values k for uracil were recorded and are reported in Table 7. Figure 11 shows the dependency of mobile phase acetonitrile content versus retention factors. For most columns uracil exhibited little retention (mean k of 0.2) between 5% and 60% acetonitrile. Above 60% acetonitrile, k (uracil) increased with acetonitrile content up to a mean value of about 1, demonstrating hydrophilic retention. The strongest HILIC characteristics were shown by Syncronis HILIC and Experimental HILIC materials.

Hypercarb material displayed both typical reversed- and HILIC-mode retention characteristics, according to the percentage of organic in the mobile phase. As illustrated in Figure 11, at acetonitrile concentrations between 60–90%, uracil retention increased as the percentage of acetonitrile increased (HILIC mode of interaction); between 10–60% acetonitrile, uracil retention decreased as the concentration of acetonitrile became greater (a reversed-phase interaction phenomenon). This dual behaviour is due to a combination of dispersive interactions between uracil-mobile phase and uracil-graphitic surface together with charge-induced interactions of uracil with the polarizable surface of the graphite (schematically shown in Figure 1e). Similarly, Acclaim HILC-10 and Acclaim Mixed Mode HILIC-1 materials exhibit "U" shaped retention versus acetonitrile curves for uracil, confirming their bimodal retention behaviour. Acclaim HILIC-10 material demonstrated stronger HILIC character than Acclaim Mixed Mode HILIC-1 and Hypercarb materials. Hypercarb material showed the strongest reversed-phase retention, suggesting a strong hydrophobicity in highly aqueous conditions.

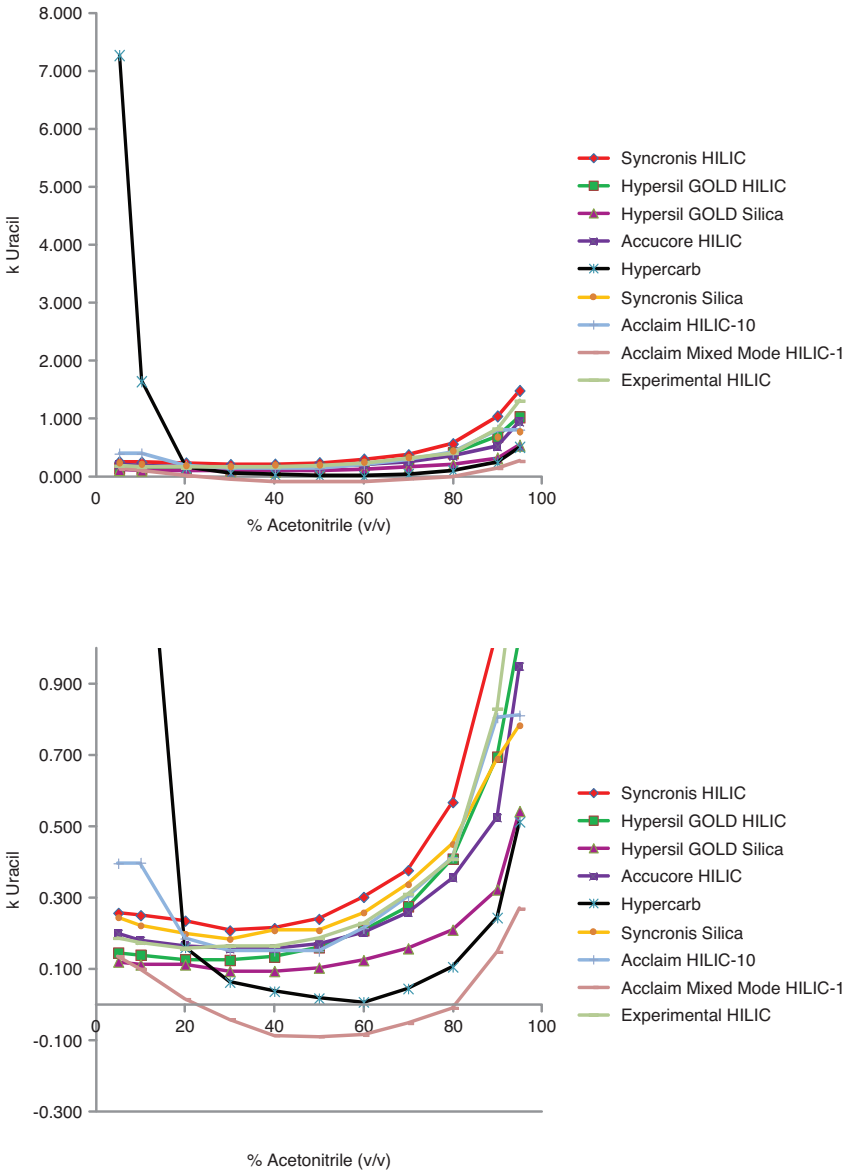


Figure 11: Effect of acetonitrile content on uracil retention. The lower graphs is the expanded version of the top graph.

MeCN%	Syncronis HILIC k' Uracil	Hypersil GOLD HILIC k' Uracil	Hypersil GOLD Silica k' Uracil	Accucore HILIC k' Uracil	Hypercarb k' Uracil	Acclaim HILIC-10 k' Uracil	Acclaim Mixed Mode HILIC-1 k' Uracil	Syncronis Silica k' Uracil	Experimental HILIC k' Uracil
95	1.488	1.032	0.543	0.950	0.513	0.812	0.269	0.784	1.312
90	1.048	0.696	0.323	0.525	0.244	0.806	0.148	0.691	0.831
80	0.568	0.409	0.210	0.356	0.105	0.414	-0.010	0.450	0.408
70	0.377	0.275	0.157	0.259	0.045	0.303	-0.053	0.338	0.311
60	0.302	0.208	0.124	0.203	0.007	0.214	-0.086	0.258	0.229
50	0.240	0.160	0.103	0.169	0.017	0.150	-0.090	0.208	0.186
40	0.214	0.134	0.094	0.156	0.037	0.150	-0.087	0.208	0.165
30	0.208	0.125	0.094	0.156	0.063	0.152	-0.042	0.184	0.165
20	0.235	0.126	0.122	0.165	0.160	0.185	0.015	0.198	0.156
10	0.250	0.139	0.122	0.180	1.649	0.398	0.100	0.222	0.173
5	0.257	0.145	0.120	0.199	7.290	0.396	0.135	0.245	0.186

Table 7. Uracil retention factors and their dependency on mobile phase acetonitrile content

Conclusion

Thermo Scientific HILIC and Hypercarb phases were characterized in terms of:

- hydrophobic 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. The degree of ion-exchange interactions had a significant influence on the shapes of these graphs, and allowed separating the HILIC stationary phases in two groups:

1. Phases containing amides, sulfonates and zwitterionic groups 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 (hydrogen-bond donors) and bare silica materials showed relatively low retention, low selectivity, and considerable ion exchange activity. These materials should be used with this in mind when analyzing acids or bases, so that the ion-exchange properties can be employed to one's advantage. Table 8 summarizes this column dichotomy.

Column Name	Phase Type	Column Group
Hypercarb (5 µm)	PGC	N/A
Hypersil GOLD HILIC (5 µm)	Polyethyleneimine	2
Hypersil GOLD Silica (5 µm)	Unbonded Silica	2
Hypersil GOLD Silica (1.9 µm)	Unbonded Silica	2
Syncronis Silica (5 µm)	Unbonded Silica	2
Accucore HILIC (2.6 µm)	Unbonded Silica	2
Acclaim Mixed Mode HILIC-1 (5 µm)	Mixed Mode Diol	2
Acclaim HILIC-10 (3 µm)	Proprietary	2
Acclaim Trinity P1 (3 µm)	NSH	2
Experimental HILIC (3 µm)	Polyacrylamide	1
Syncronis HILIC (5 µm)	Zwitterion	1

Table 8: HILIC stationary phases two main groups

		Retention Time (min)			Test Parameters			Uridine		5-Methyluridine	
Column	Injection No.	Toluene	5-methyluridine (5MU)	Uridine (U)	k U	k 5MU	α (CH_2) (k U/k 5MU)	Asymmetry	Area	Asymmetry	Area
Syncronis HILIC	1	2.073	8.624	11.626	4.608	3.160	1.458	1.178	2288	1.189	1882
	2	2.047	8.911	12.161	4.941	3.353	1.473	1.70	2253	1.182	1870
	3	2.044	9.011	12.334	5.034	3.409	1.477	1.166	2252	1.179	1868
	4	2.036	9.188	12.652	5.214	3.513	1.484	1.160	2258	1.176	1878
	5	2.031	9.208	12.692	5.249	3.534	1.485	1.166	2267	1.177	1878
	6	2.032	9.239	12.743	5.271	3.547	1.486	1.164	2265	1.178	1883
	Average	2.044	9.030	12.743	5.053	3.419	1.477	1.256	2263.833	1.180	1876.500
	Std Dev	0.016	0.237	0.429	0.254	0.148	0.011	0.218	13.318	0.005	6.189
	%RSD	**0.77**	**2.62**	**3.47**	**5.03**	**4.33**	**0.72**	**17.34**	**0.59**	**0.41**	**0.33**

Column	Injection No.	Acetone	5-Methyluridine (5MU)	Uridine (U)	k U	k 5MU	α (CH_2) (k U/k 5MU)	Asymmetry	Area	Asymmetry	Area
Hypercarb	1	2.614	6.416	4.613	0.765	1.454	0.526	1.318	2326	0.992	1882
	2	2.614	6.413	4.610	0.764	1.453	0.525	1.311	2321	0.996	1880
	3	2.614	6.413	4.611	0.764	1.453	0.526	1.313	2323	0.996	1882
	4	2.615	6.417	4.614	0.764	1.454	0.526	1.311	2328	0.993	1885
	5	2.613	6.412	4.610	0.764	1.454	0.526	1.314	2324	0.989	1881
	6	2.614	6.398	4.603	0.761	1.448	0.526	1.309	2320	0.993	1879
	Average	2.614	6.412	4.610	0.764	1.453	0.526	1.313	2323.667	0.993	1881.500
	Std Dev	0.001	0.007	0.004	0.001	0.003	0.000	0.003	3.011	0.003	2.074
	%RSD	**0.02**	**0.11**	**0.08**	**0.18**	**0.18**	**0.03**	**0.24**	**0.13**	**0.27**	**0.11**

		Retention Time (min)			Test Parameters			Adenosine		Vidarabine	
Column	Injection no.	Toluene	Adenosine (A)	Vidarabine (V)	k A	k V	α V/A (k V/k A)	Asymmetry	Area	Asymmetry	Area
Syncronis HILIC	1	2.107	9.174	11.984	3.354	4.688	1.398	1.204	980	1.182	1329
	2	2.043	9.691	12.751	3.744	5.241	1.400	1.198	981	1.171	1326
	3	2.008	9.969	13.184	3.965	5.566	1.404	1.193	980	1.165	1325
	4	1.986	10.136	13.434	4.104	5.764	1.405	1.188	979	1.158	1325
	5	1.973	10.239	13.599	4.190	5.893	1.406	1.185	980	1.155	1326
	6	1.966	10.302	13.695	4.240	5.966	1.407	1.182	980	1.154	1327
	Average	2.014	9.919	13.108	3.933	5.520	1.403	1.192	980.000	1.164	1326.333
	Std Dev	0.053	0.426	0.646	0.335	0.484	0.004	0.008	0.632	0.011	1.506
	%RSD	**2.66**	**4.29**	**4.93**	**8.52**	**8.76**	**0.26**	**0.70**	**0.06**	**0.93**	**0.11**

Column	Injection no.	Acetone	Adenosine (A)	Vidarabine (V)	k A	k V	α V/A (k V/k A)	Asymmetry	Area	Asymmetry	Area
Hypercarb	1	2.610	20.034	35.093	6.676	12.446	1.864	1.684	666	1.545	558
	2	2.609	20.057	35.078	6.688	12.445	1.861	1.673	670	1.594	536
	3	2.609	20.057	35.076	6.688	12.444	1.861	1.685	710	1.525	418
	4	2.609	20.072	35.225	6.693	12.501	1.868	1.696	692	1.348	401
	5	2.609	20.121	35.258	6.712	12.514	1.864	1.665	697	1.496	477
	6	2.608	20.115	35.142	6.713	12.475	1.858	1.709	740	1.715	428
	Average	2.609	20.076	35.145	6.695	12.471	1.863	1.685	695.833	1.537	469.667
	Std Dev	0.001	0.035	0.079	0.015	0.031	0.003	0.016	27.294	0.120	65.387
	%RSD	**0.02**	**0.17**	**0.22**	**0.22**	**0.25**	**0.18**	**0.93**	**3.92**	**7.84**	**13.92**

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