The CHROMacademy Essential Guide to

Presented by LCGC's CHROMacademy powered by crawford scientific

Understanding HPLC Column Characterization and Selection

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LC GC's CHROMacademy

powered by crawford scientific

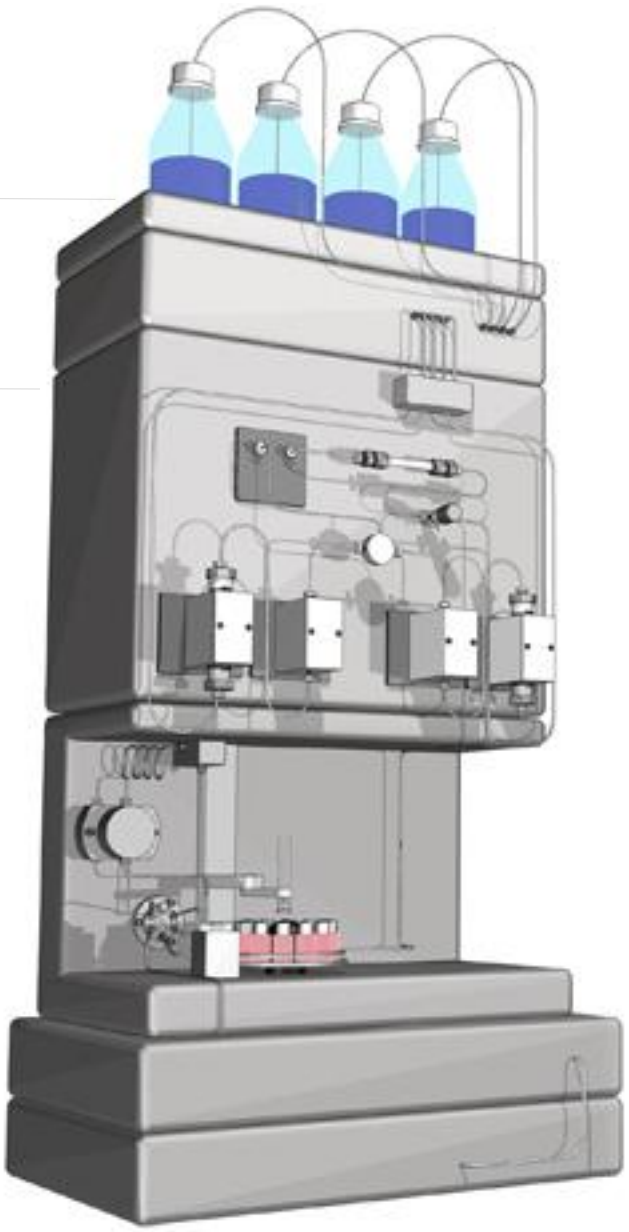
Interactive HPLC Troubleshooter

fixing your system is now child's play

Step 1: Select your chromatographic symptoms

Step 2: Select your instrument symptoms

Step 3: Get your solution!



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Aims & Objectives

- Essential characteristics of an HPLC stationary phase
- Review of bonded phase chemistry
- Why characterize HPLC columns?
- Column classification databases
- Common classification tests
- Interpreting classification results
- Using column classification to aid column selection
- Relating column properties to analyte characteristics
- Similar and orthogonal phases
- Column classification – the future

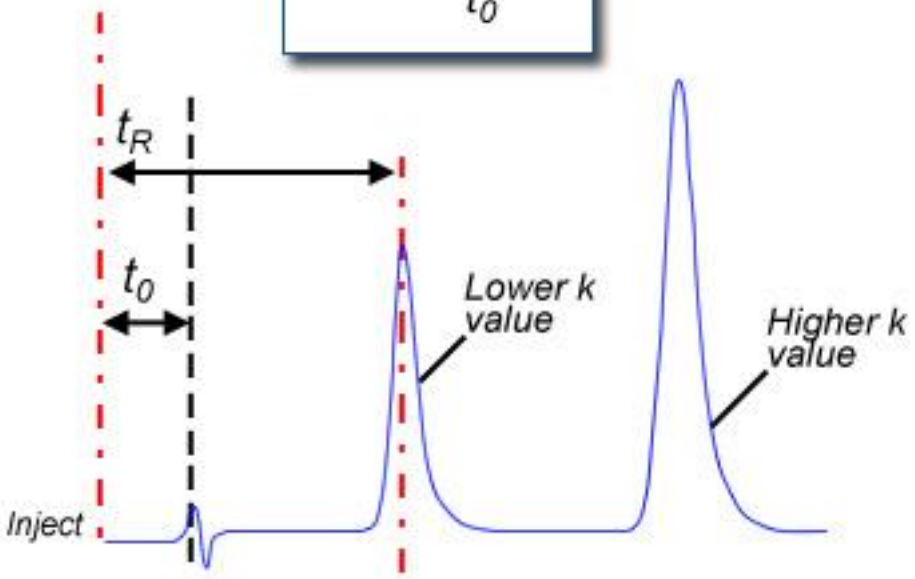
Column Classification – Why?

- Define the essential characteristics of stationary phases in terms of retentivity but more especially selectivity
- Derive a set of test probes which highlight differences between these properties
- Use the data generated to characterize all columns available
- Use chemometrics to find a useful way to display this data for easy column comparison
- Rank columns on similarity / difference based on a specific property or on overall performance
- Identify columns which have a dominant (helpful) characteristic for method development
- Gain better insight into problems / issues by relating analyte properties to column characteristics (column interactions)

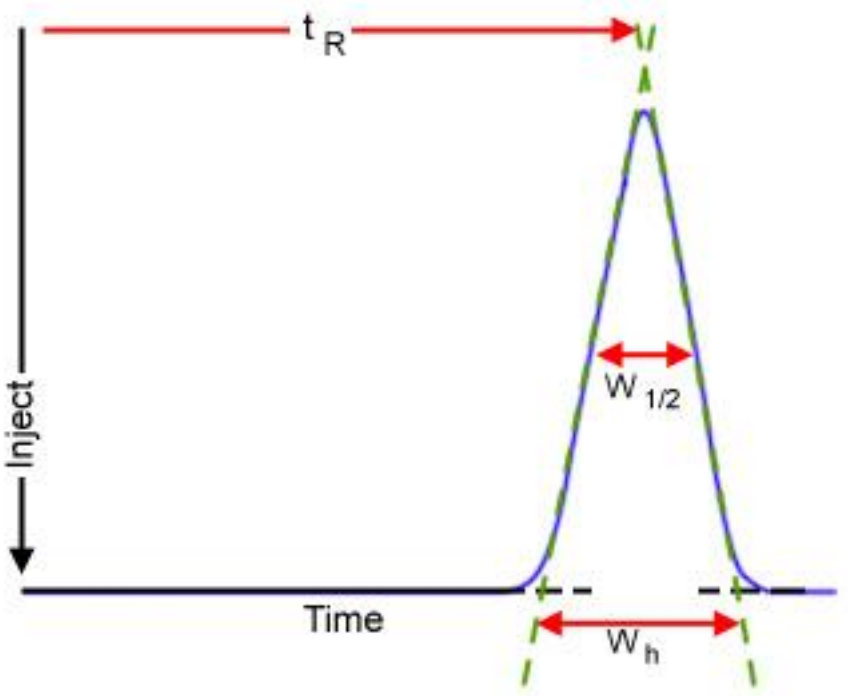
Measuring Column Performance (I)

Retention Factor (k)

$$k = \frac{(t_R - t_0)}{t_0}$$



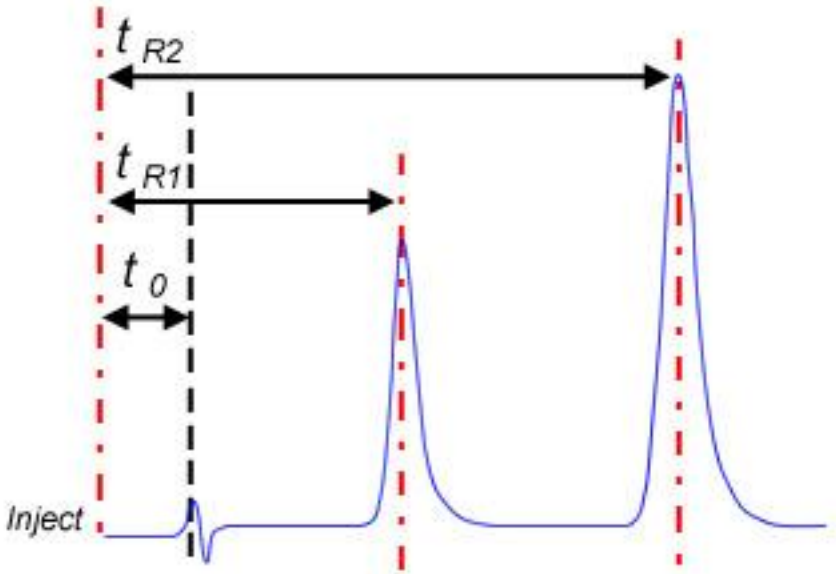
Efficiency (N)



$$N = 16\left(\frac{t_r}{W_b}\right)^2 = 5.54\left(\frac{t_r}{W_h}\right)^2$$

Measuring Column Performance (II)

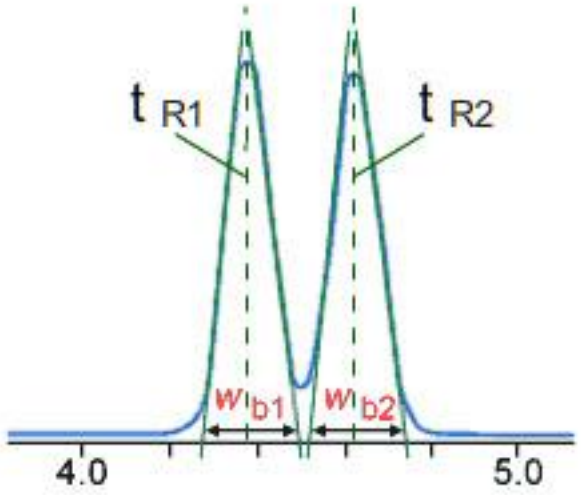
Selectivity (α)



$$\alpha = \frac{k'_2}{k'_1} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

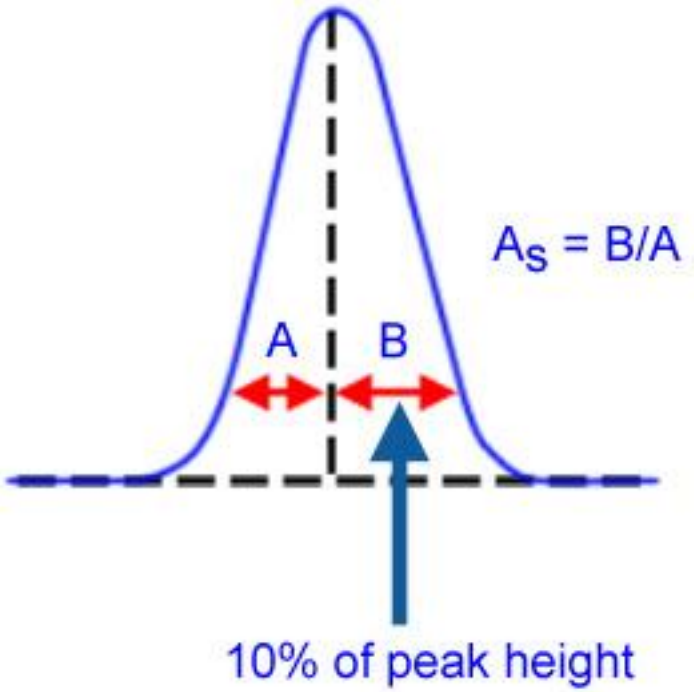
Resolution (R)

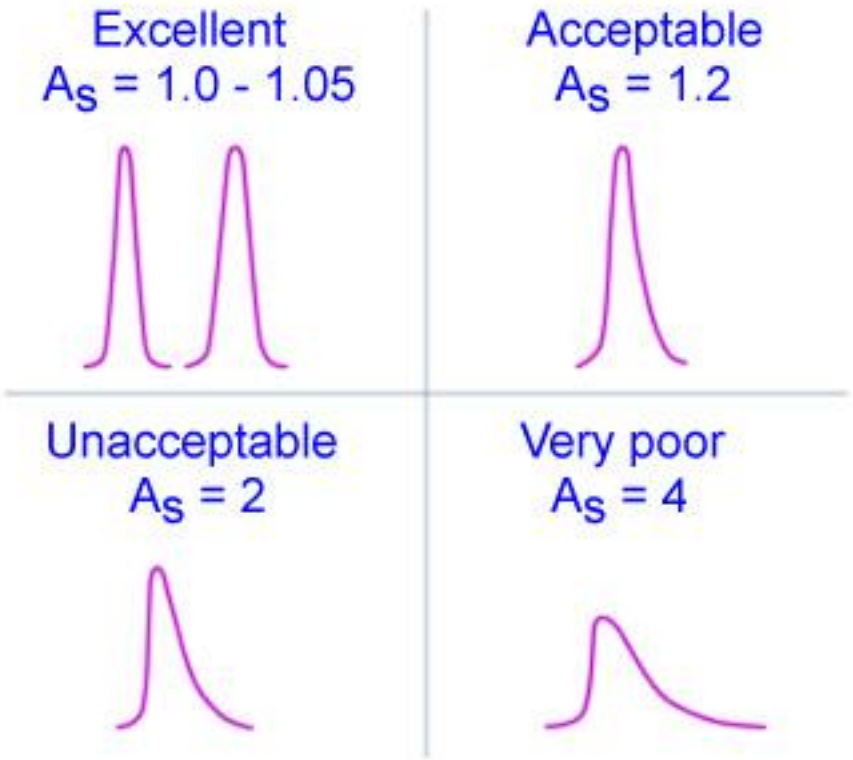
$$R_s = \frac{(t_{R2} - t_{R1})}{(w_{b1} + w_{b2})/2} = \frac{2(t_{R2} - t_{R1})}{w_{b1} + w_{b2}}$$



Measuring Column Performance (III)

Peak Asymmetry (A_s)





C18 HPLC Column Essential Properties (I)

Bonded phase

- Standard octadecylsilyl alkyl silane
- Polar Embedded C18 (with imide, carbamate etc. spacer)
- Mixture of C18 and shorter alkyl chains
- Nature of the silane substituents (e.g di-isobutyl silane)
- Carbon loading
- Carbon loading to silica surface area ratio (phase density)

C18 HPLC Column Essential Properties (II)

Nature of the base silica

- Sol or sil-gel particle
- Type I or Type II silica
- Silica metal ion content
- Totally porous, polymeric or superficially porous support
- Pure silica or organic / inorganic hybrid
- Spherical or irregular silica particle
- Particle size and particle size distribution
- Pore size
- Surface area
- Deactivation / nature of the end-capping reagent

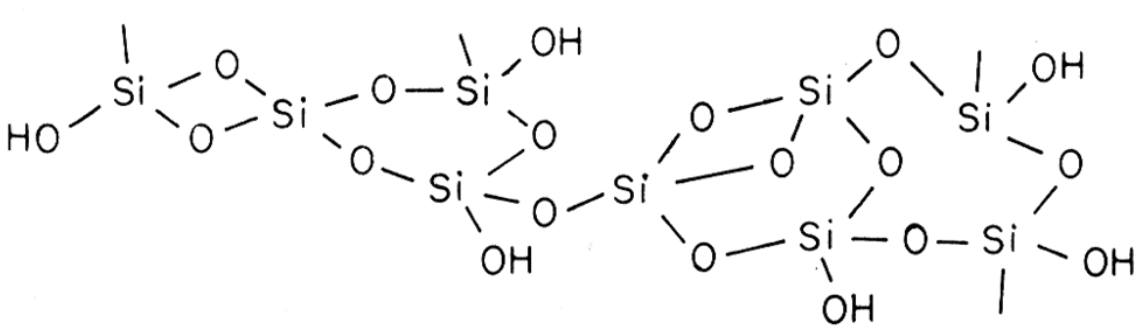
C18 HPLC Column Essential Properties (III)

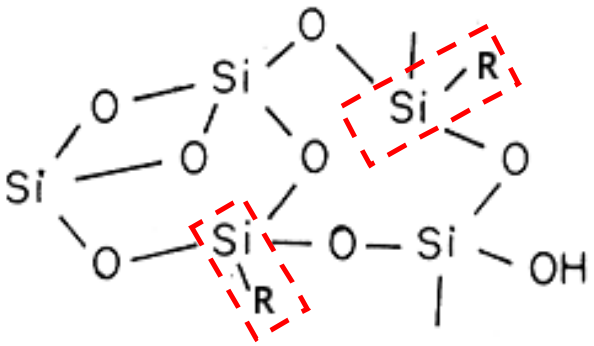
Column Format

- Column Length
- Internal diameter
- Nature of the material used for column construction
- Metal passivation technique
- Interior tubing surface polishing
- Nature of the frit and spreaders used in the column end fittings (porosity and material of construction)
- Packing methodology (packing pressure, solvents used etc.)

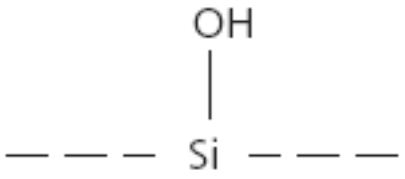
Silica as a support material (I)

Nature of base silica

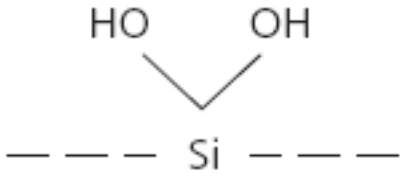




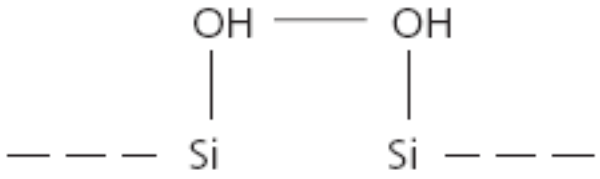
Silanol species



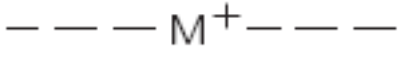
Free silanol



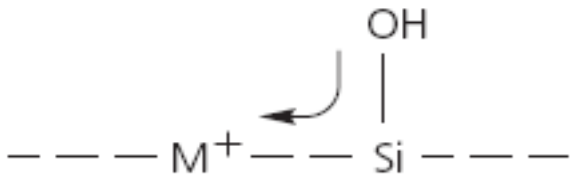
Geminal silanols



Associated silanols

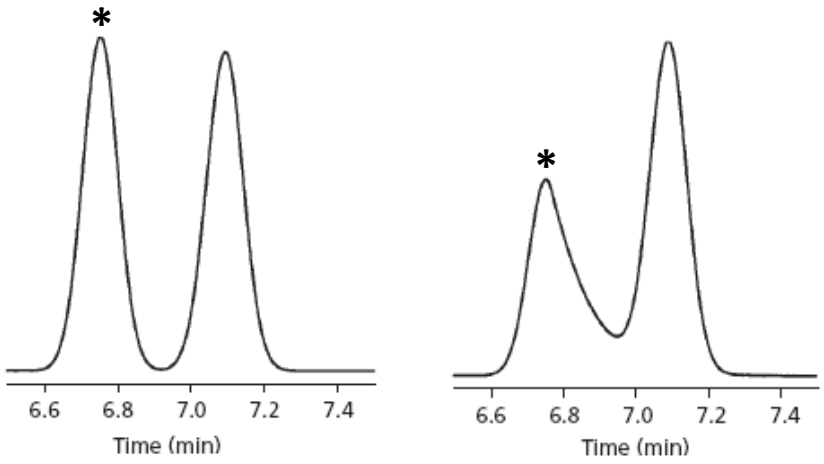


Surface metal



Internal metal (activated silanol)

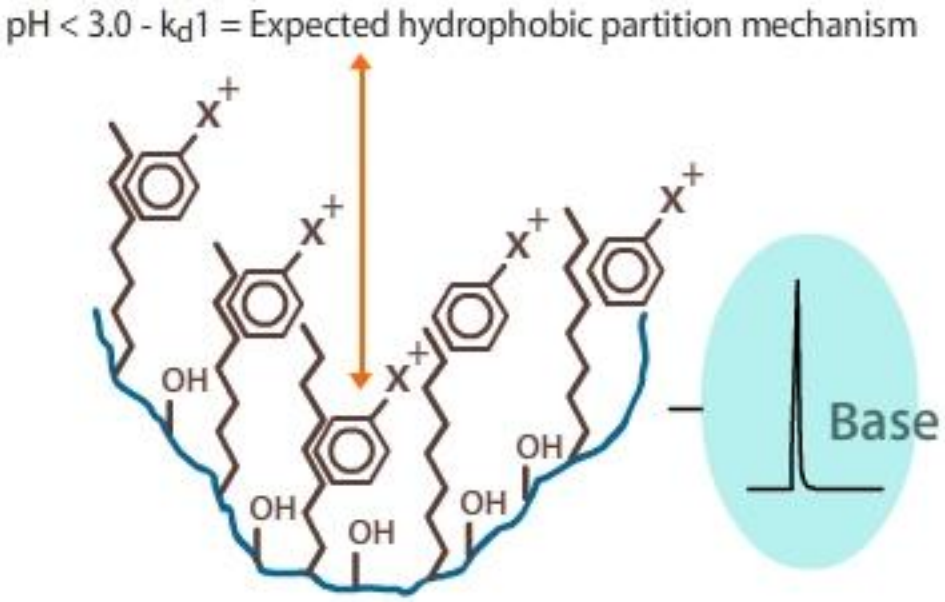
Silica as a support material (II)

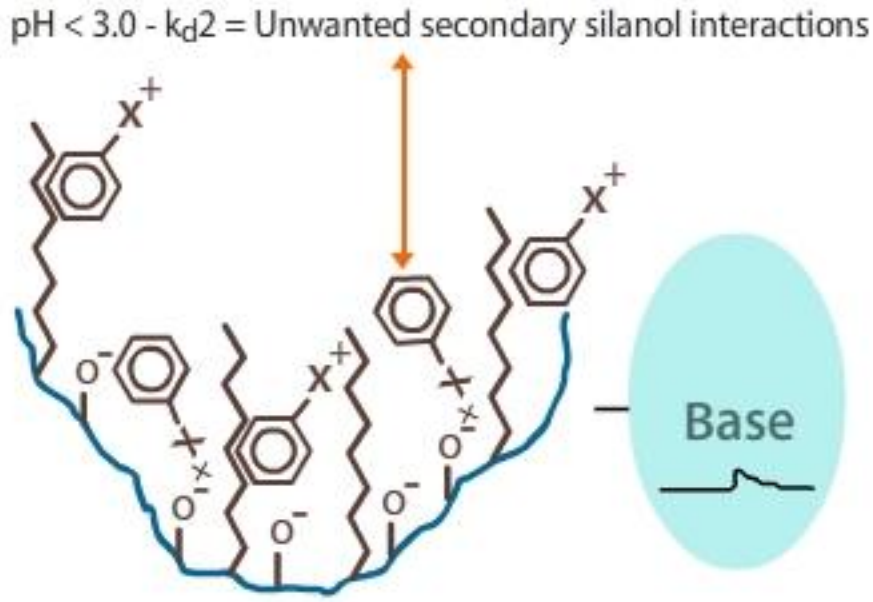


Type I and II Silica

Basic, polar analyte (*) analysed using Type I (right) and Type II (left) silica under reversed phase conditions (45% MeCN / 55% 0.1%TFA, pH 2.1, 35^{o}C)

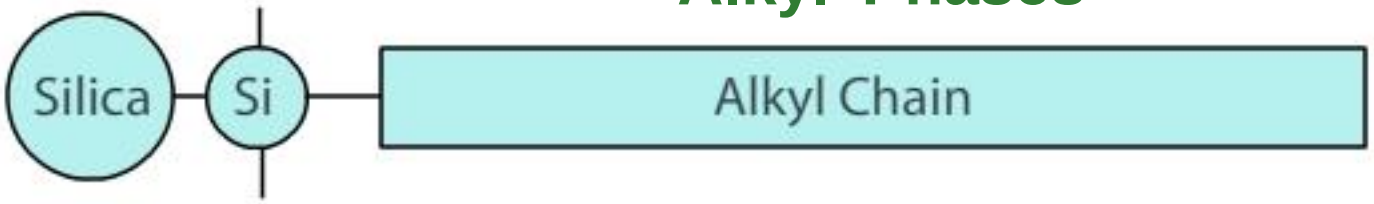
pH Dependence





Common bonded phase types (I)

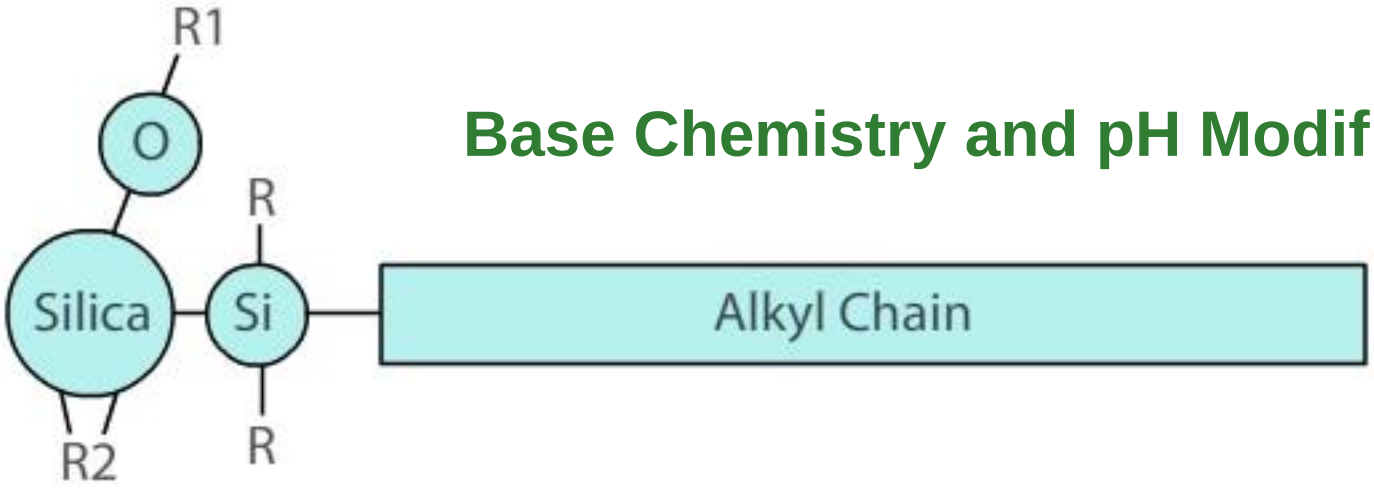
‘Alkyl’ Phases



R = Me

Alkyl chain = C_2 to C_{18} typical although $C_{30(+)}$ are also available

Base Chemistry and pH Modifications



R = OH, Me, isopropyl, isobutyl

R1 = End capping reagent - $SiMe_3$, SiI_3, $SiBr_3$, Polar group

R2 = Bridging group - Ethyl typically

Common bonded phase types (II)

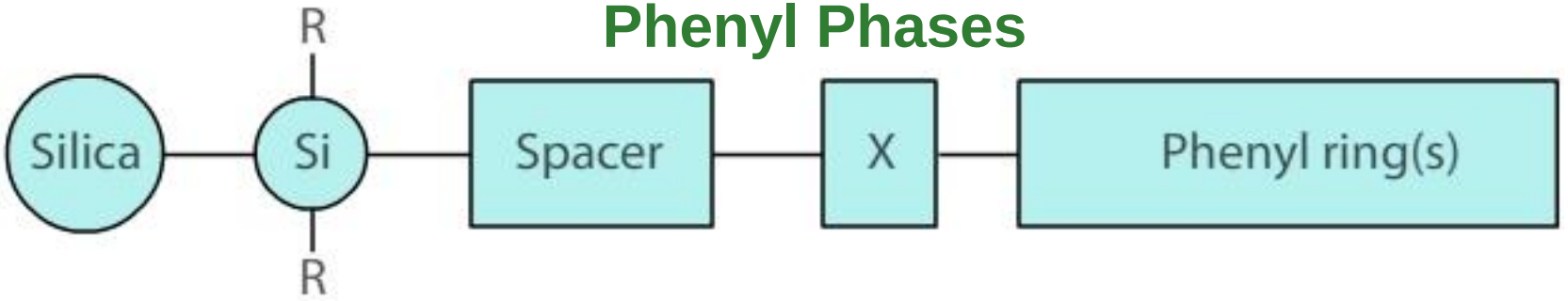
Polar Embedded Phases

Polar group = amide, carbamate, urea, sulphonamide, alkyl or phenyl ether

Spacer = short chain alkyl

Alkyl chain = C8 to C18 typically

Phenyl Phases



Silica = Pure or traditional

R = OH, Me, isopropyl

Spacer = Typically C_0 to C_6

Phenyl rings = One or two

Endcapping = yes or no

Bonding = Monomeric or polymeric

X = Heteroatom or CH_2

Key Column Classification Initiatives

Tanaka et. al.

K. Kimata, K. Iwaguchi, S. Onishi, K. Jinno, R. Eksteen, K. Hosoya, M. Arki, N. Tanaka, *J. Chromatogr. Sci.* **27** (1989) 721.

Euerby & Petersson

M. R. Euerby, P. Petersson, J. Chromatogr. A 994 (2003), p. 13 – 36

USP – Working Group on HPLC Columns

Pharmacopeial Forum Vol. 31(2) [Mar.–Apr. 2005]

PQRI – Snyder, Dolan et. al.

N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171-193.

Important Column Variables

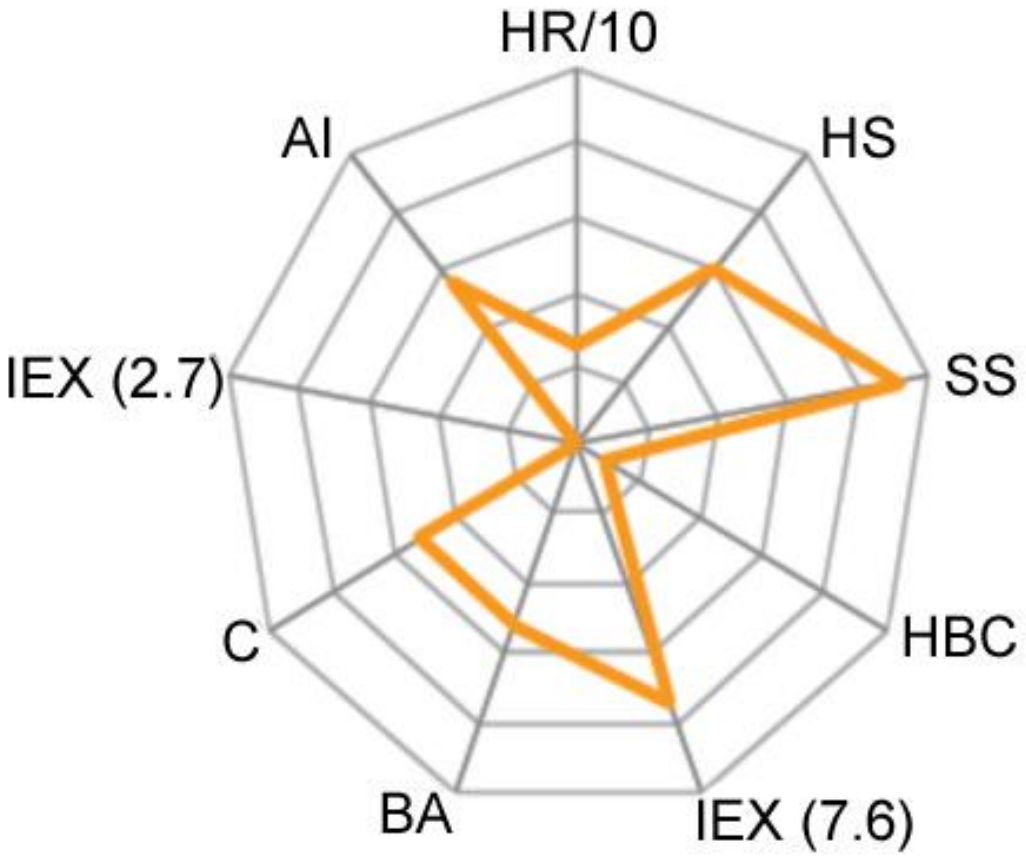


Figure courtesy of ThermoFisher Scientific, Runcorn, UK

Variables

HR – hydrophobic retention

HS – hydrophobic selectivity

SS – steric selectivity

HBC – hydrogen bonding capacity

BA – base activity

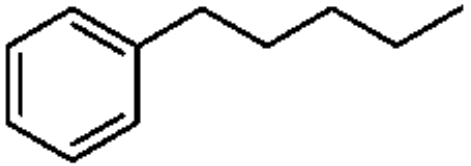
C – chelation

IEX – ion exchange capacity at pH 2.6 and 7.6

AI – acid interaction

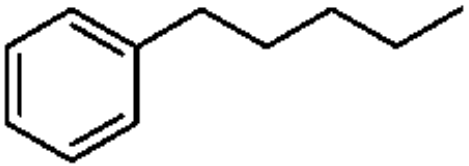
Tanaka Probes(I)

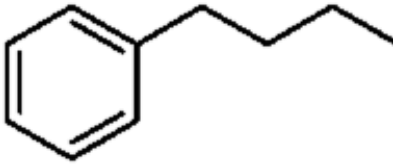
Retention Factor (k_{PB}) - retention factor of pentylbenzene (k_{PB}) / surface coverage & ligand density (methanol as t_0)



Conditions: MeO:H_2O (80:20, v/v), 1.0 ml/min, 40 ^oC, 5 μl injection of pentylbenzene (0.6 mg/ml)

Hydropbobic Selectivity (α_{CH2}) – selectivity between pentylbenzene and butylbenzene / further measure of ligand density ($\alpha_{CH2} = k_{PB} / k_{BB}$)





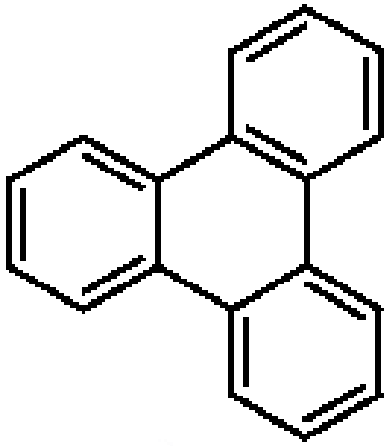
Pentybenzene logP (o/w) : 4.90

Butylbenzene logP (o/w) : 4.27

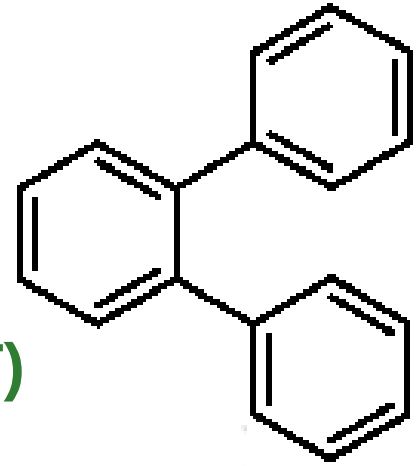
Conditions: MeOH–H_2O (8:2, v/v), 1.0 ml/min, 40 ^oC, individual 5 μl injection of pentylbenzene (0.6 mg/ml) and butylbenzene (0.3 mg/ml)

Tanaka Probes (II)

Shape Selectivity ($\alpha_{T/O}$) –ability of the phase to discriminate between planar structures (triphenylene) and those with greater spatial volume (o-terphenyl) / reflects ligand spacing & shape functionality of end capping reagent ($\alpha_{T/O} = k_T / k_O$)



triphenylene (T)



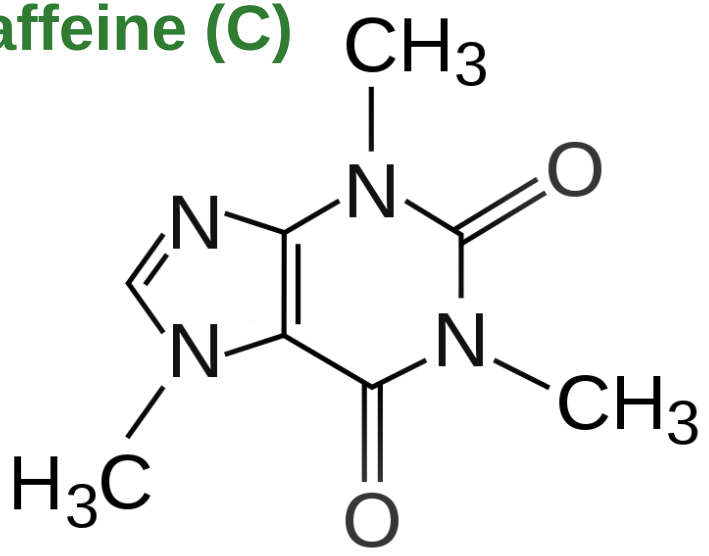
o-terphenyl (O)

Conditions: MeOH–H_2O (80:20, v/v), 1.0 ml/min, 40 ^oC, individual 5 μl injection of o-terphenyl (0.05 mg/ml) and triphenylene (0.05 mg/ml)

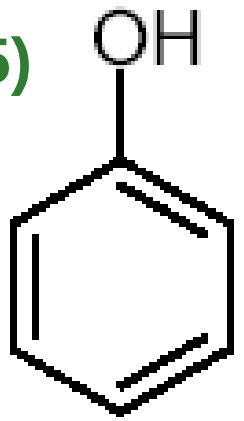
Tanaka Probes (III)

Hydrogen bonding capacity,($\alpha_{C/P}$) – selectivity between caffeine and phenol / descriptor of the columns ability to hydrogen bond with a solute / reflects the number of available silanol groups & nature and degree of end-capping ($\alpha_{C/P} = k_C / k_P$)

caffeine (C)



phenol (P)
(pKa = 9.95)

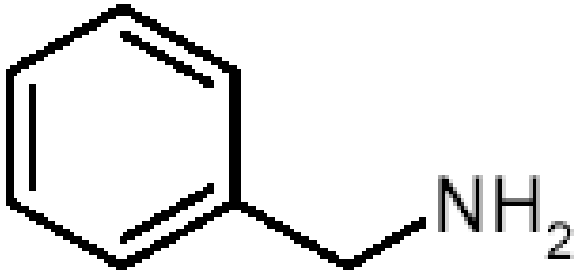


Conditions: MeOH–H_2O (3:7, v/v), 1.0 ml/min, 40 ^oC, individual 5-μl injections of phenol (1 mg/ml) and caffeine (0.5mg/ml).

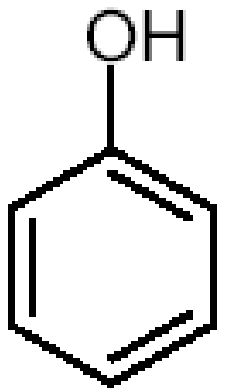
Tanaka (IV)

Total ion-exchange capacity, ($\alpha_{B/P}$ pH 7.6) - selectivity between benzylamine and phenol at a mobile phase pH of 7.6 / reflects the total silanol activity (total ion exchange capacity) of the column ($\alpha_{B/P} = k_B / k_P$ (pH 7.6))

Benzylamine (B)
(pKa 9.33)



phenol (P)
(pKa = 9.95)

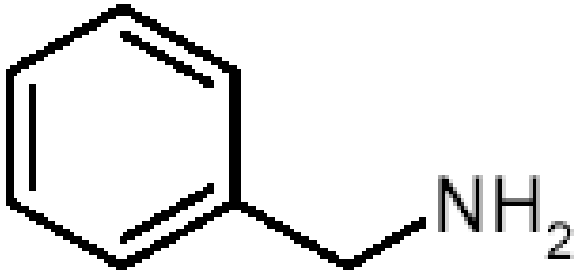


Conditions: 20 m*M* KH_2PO_4 , pH 7.6, in MeOH–H_2O (30:70), 1.0 ml/min, 40 °C, individual 5-µl injections of phenol and benzylamine HCl both at 0.5 mg/ml.

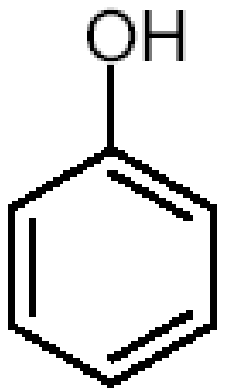
Tanaka (V)

Total ion-exchange capacity, ($\alpha_{B/P}$ pH 2.7) - selectivity between benzylamine and phenol at a mobile phase pH of 2.7 / reflects the acidity of the silanol surface
($\alpha_{B/P} = k_B / k_P$ (pH 2.7))

Benzylamine (B)
(pKa 9.33)

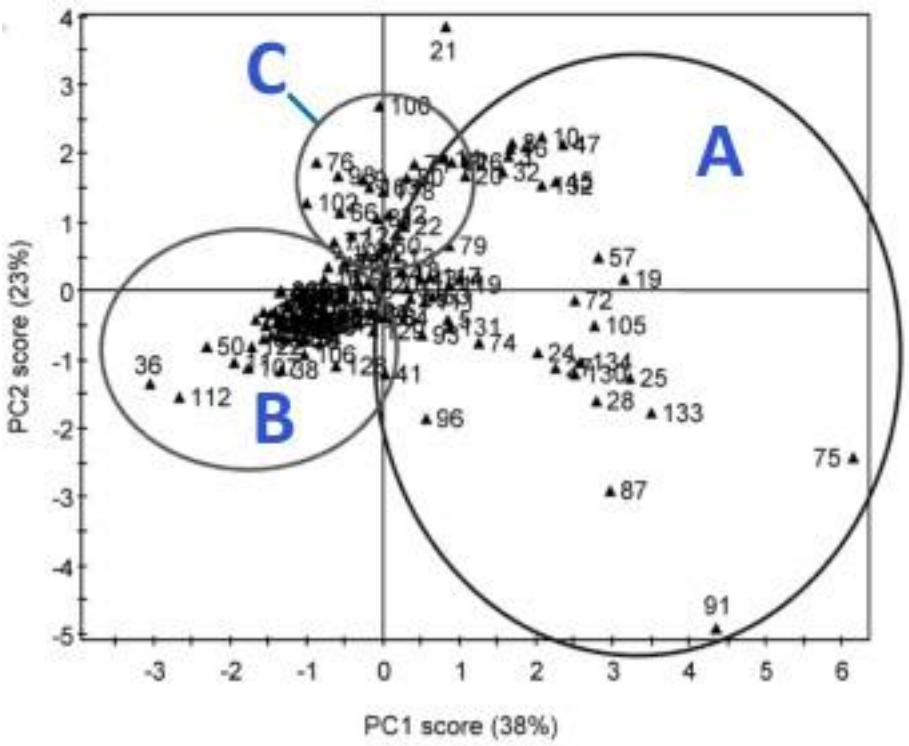


phenol (P)
(pKa = 9.95)



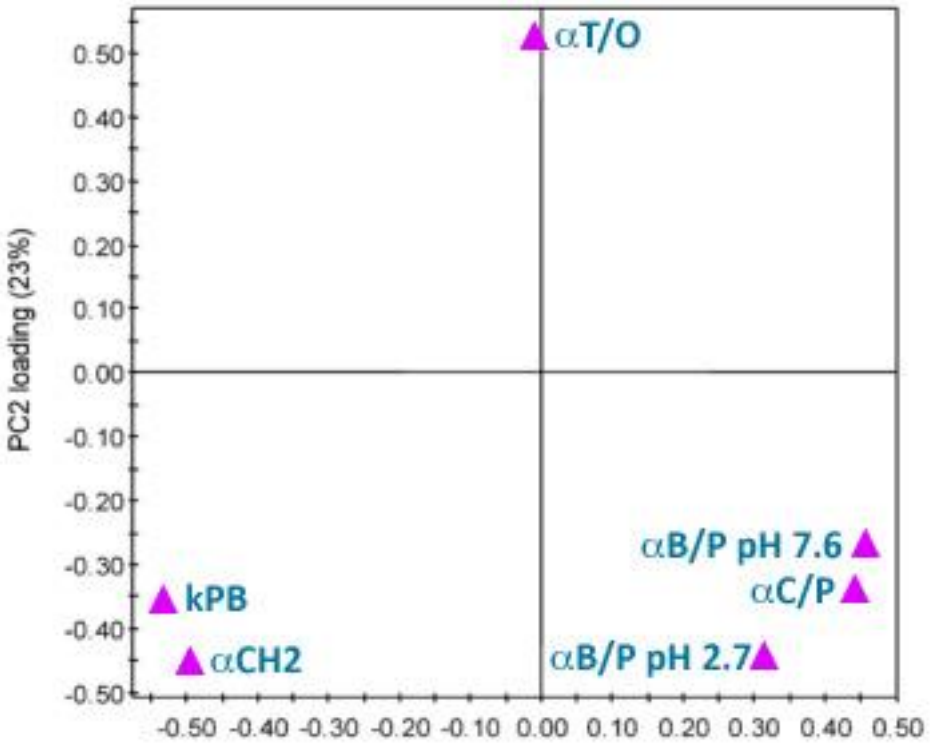
Conditions: 20 m*M* KH_2PO_4 , pH 2.7, in MeOH–H_2O (30:70), 1.0 ml/min, 40 °C, individual 5-µl injections of phenol and benzylamine HCl both at 0.5 mg/ml.

Chemometric Comparison (PCA)



PC1 and 2 score plot for 125 silica phases

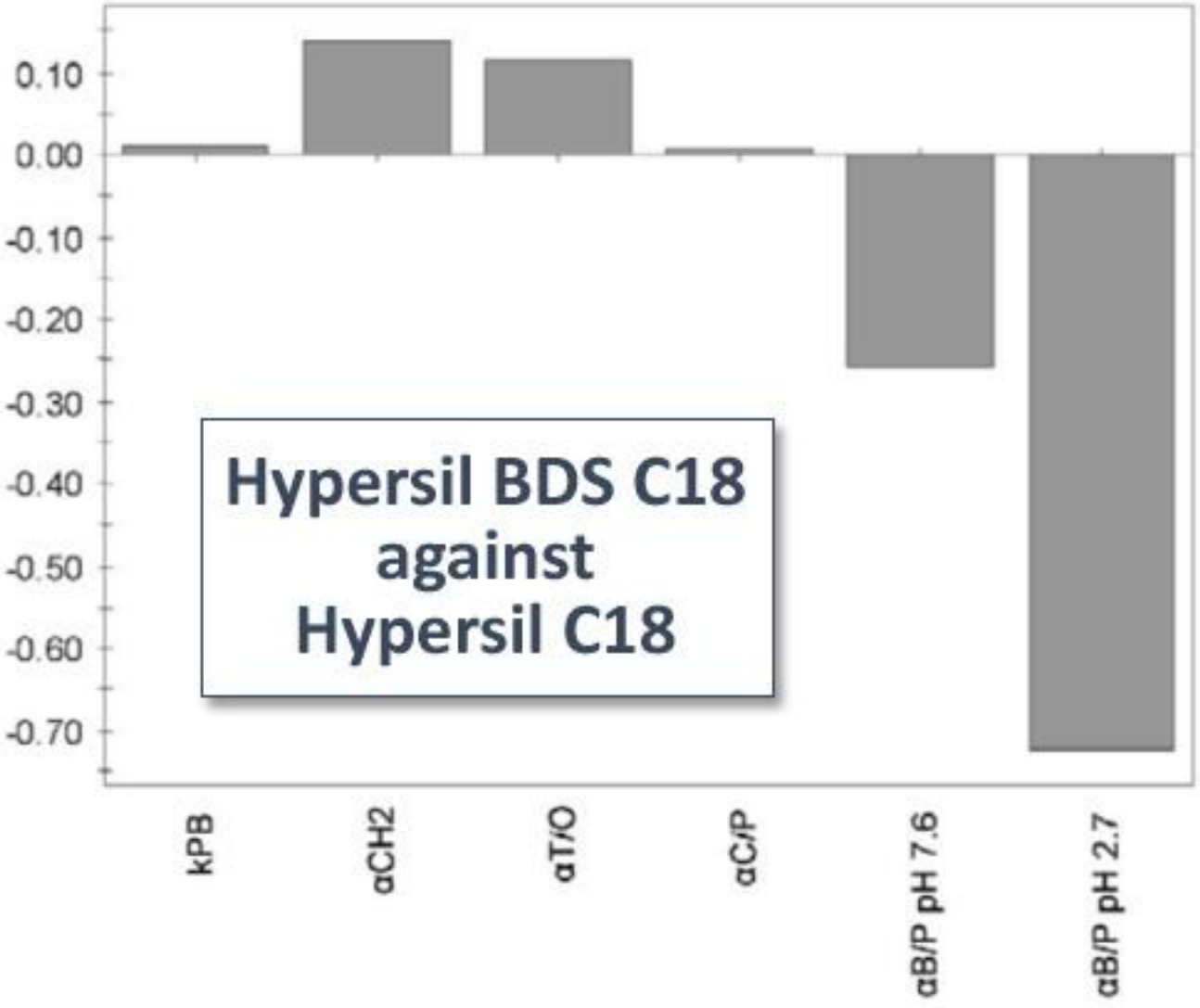
- *A - mostly non-C18 and traditional acidic (type A) C18 silica phases*
- *B - mostly non-acidic (type B)*
- *C - polar embedded phases*



PC1 and 2 loading plot for all columns excluding non-silica and amino

Chemometric Comparison (PCA)

Principal component contribution plot for two C18 columns using Tanaka probes

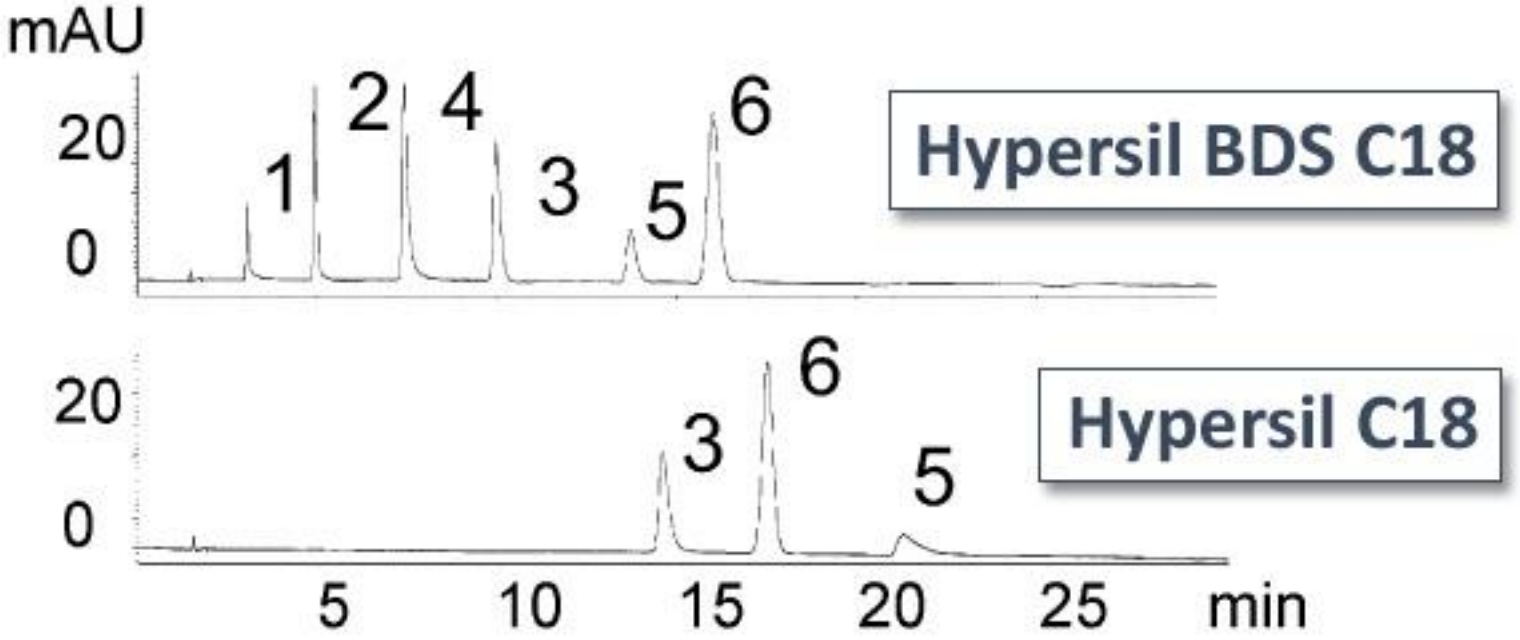


*M. R. Euerby, P. Petersson, J. Chromatogr. A **994** (2003)*

Chemometric Comparison (PCA)

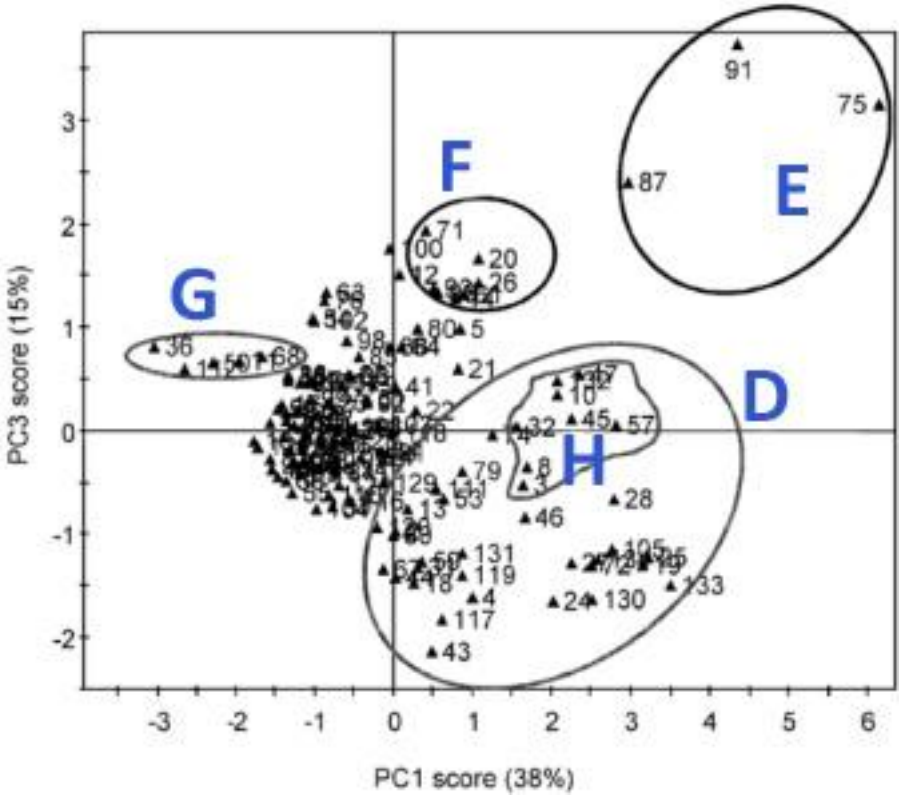
20 mM KH PO , pH 2.7, in MeOH–H_2O
(3.3:96.7, v/v), 1.0 ml/min, 60 °C, 5 µl hydrophilic base test mixture, detection at 210nm.

1 Nicotine 2 Benzylamine 3 Terbutaline 4 Procainamide
5 Salbutamol 6 Phenol



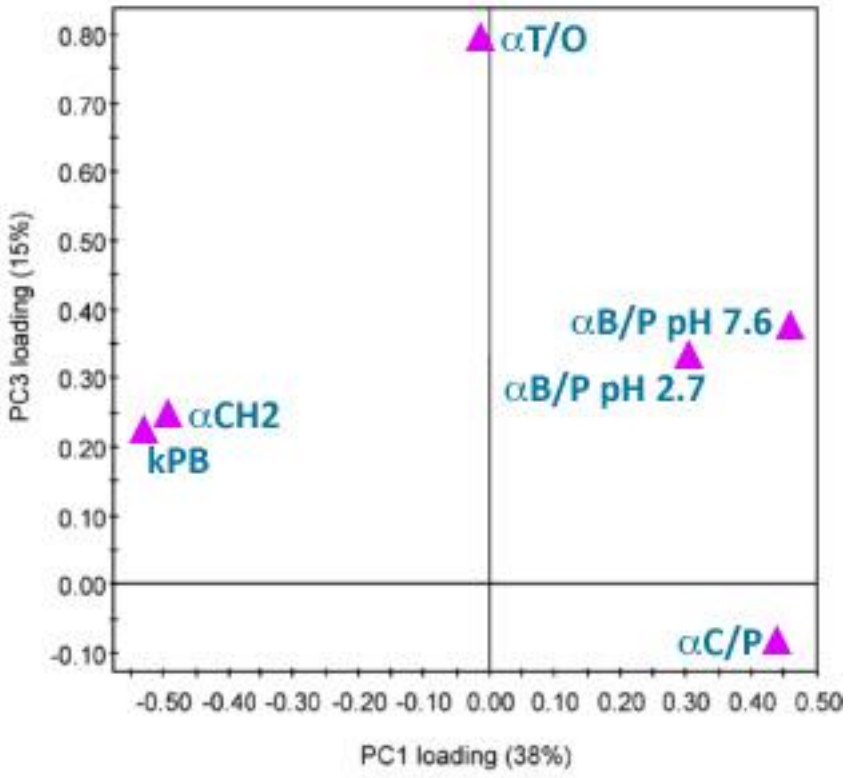
*M. R. Euerby, P. Petersson, J. Chromatogr. A **994** (2003)*

Further Chemometric Comparison (PCA)



PC1 and 3 score plot for 125 silica phases:

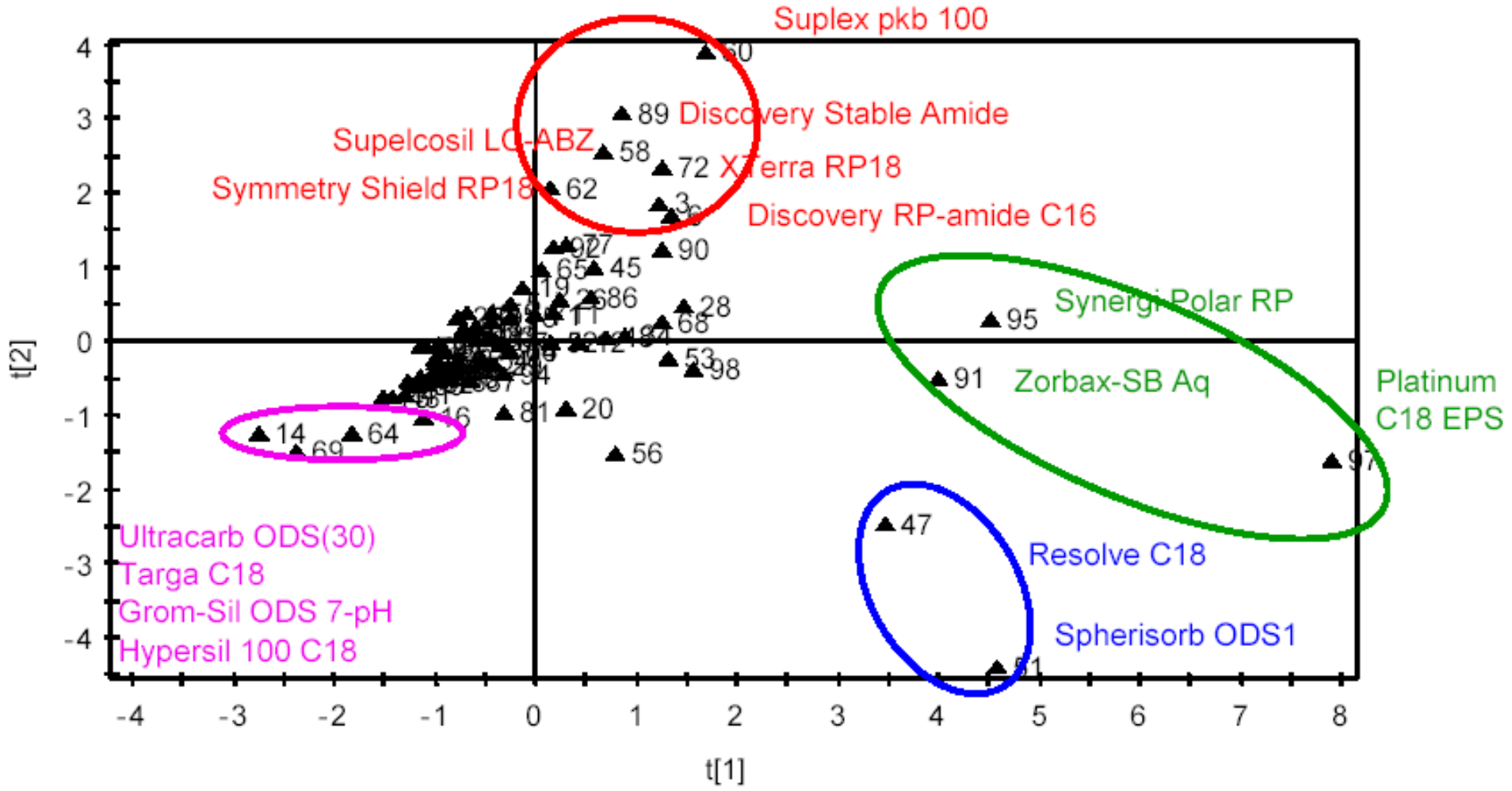
- *D – non C18 phases*
- *E – acidic phases*
- *F – perfluoro phases*
- *G – highly hydrophobic phases*
- *H – cyano phases*



PC1 and 3 loading plot for all columns excluding non-silica and amino

*M. R. Euerby, P. Petersson, J. Chromatogr. A **994** (2003)*

PCA – Practically Speaking!



*M. R. Euerby, P. Petersson, J. Chromatogr. A **994** (2003)*

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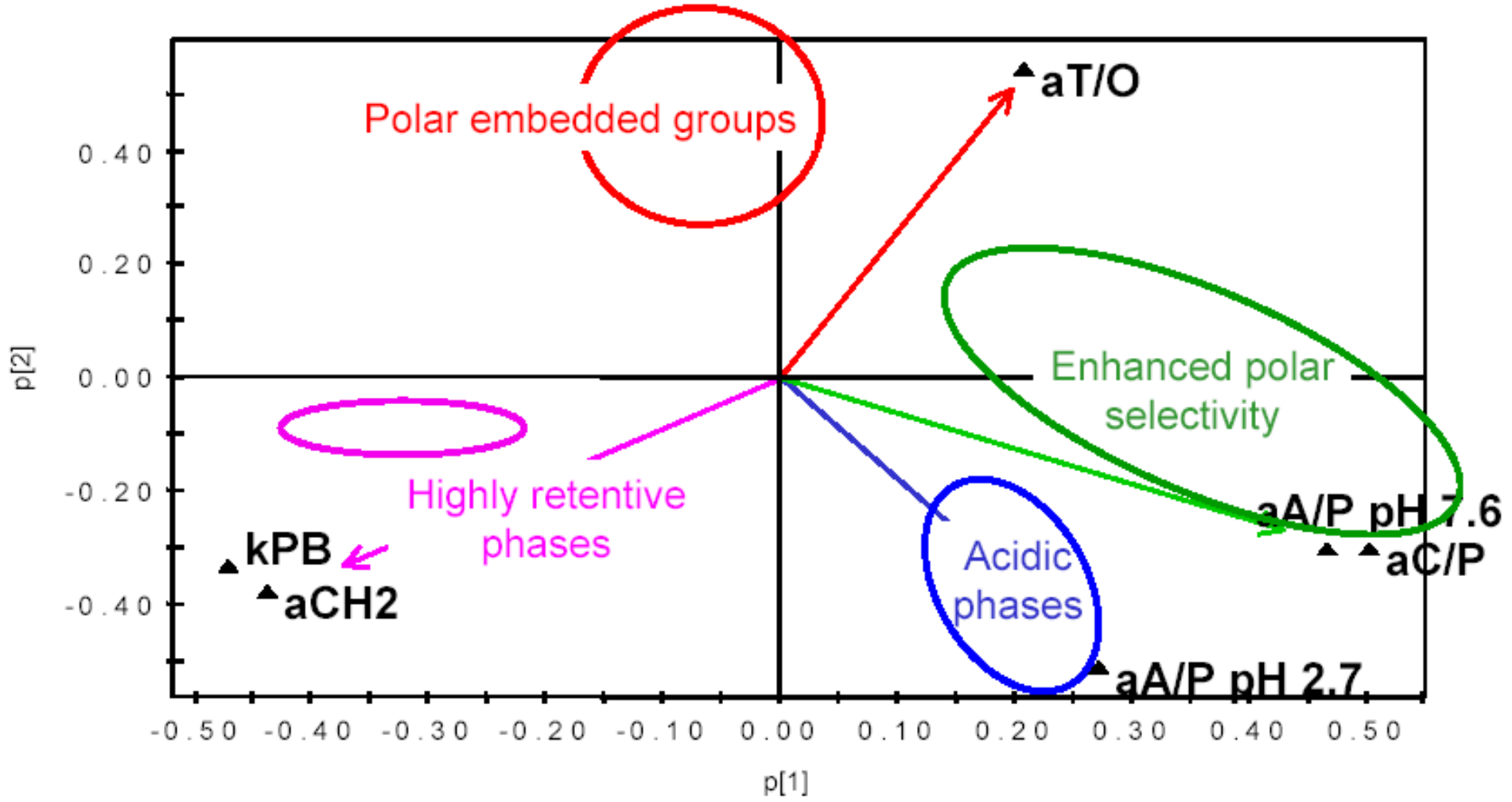
PCA – Practically Speaking!



*M. R. Euerby, P. Petersson, J. Chromatogr. A **994** (2003)*

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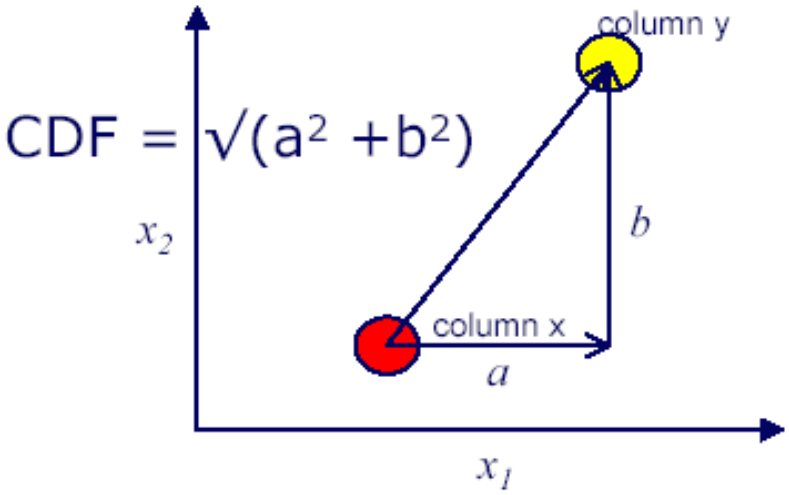
PCA – Impractically Speaking!

- PCA - not practical for everyone
- Columns have continuum of characteristics
- Columns may be on the ‘cusp’ of two classes
- Defining variable may have no relevance to the separation at hand
- Need to be able to compare columns without assigning them to ‘classes’
- Need to be able to equate differences in specific column variables to analyte structure / physico-chemical properties

Quantifying Similarity & Orthogonality



Similar Columns

CDF = 0.142

Column 1: Hypersil Gold C18
Column 2: XTerra MS C18

	Parameters					
Weightings	kPB (1.00)	aCH2 (1.00)	aTO (1.00)	aCP (1.00)	aBP76 (1.00)	aBP27 (1.00)
	2.79	1.44	1.18	0.49	0.41	0.12
	3.52	1.42	1.26	0.42	0.35	0.1

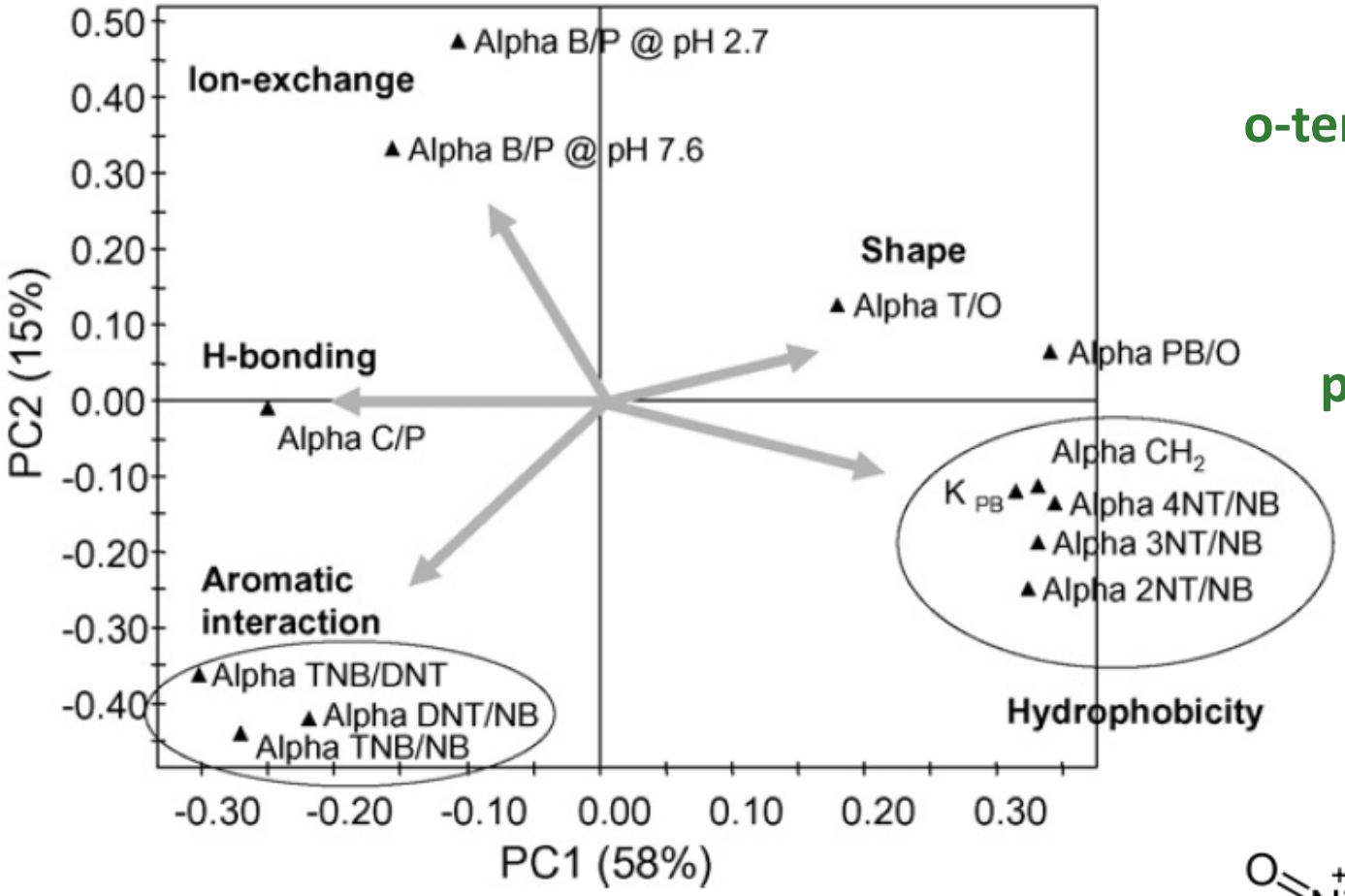
Orthogonal Columns

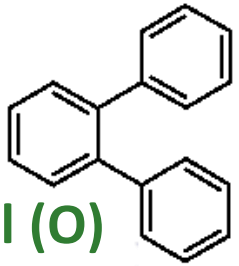
CDF = 0.643

Column 1: Hypersil Gold C18
Column 2: Primesep B

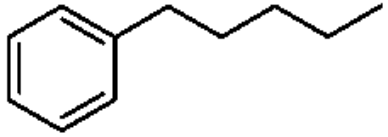
	Parameters					
Weightings	kPB (1.00)	aCH2 (1.00)	aTO (1.00)	aCP (1.00)	aBP76 (1.00)	aBP27 (1.00)
	2.79	1.44	1.18	0.49	0.41	0.12
	1.78	1.38	2	0.56	0.21	-0.03

Further Classification – 'Phenyl' phases

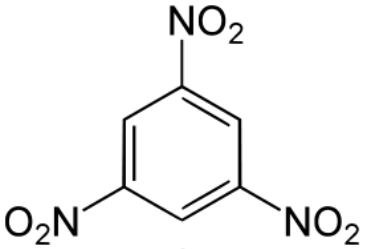




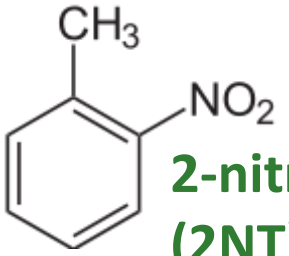
o-terphenyl (O)



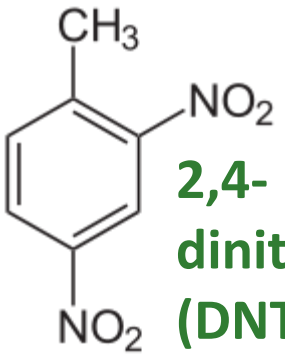
pentylbenzene (PB)



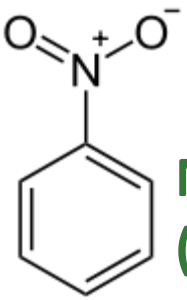
1,3,5-trinitrobenzene (TNB)



2-nitrotoluene (2NT)



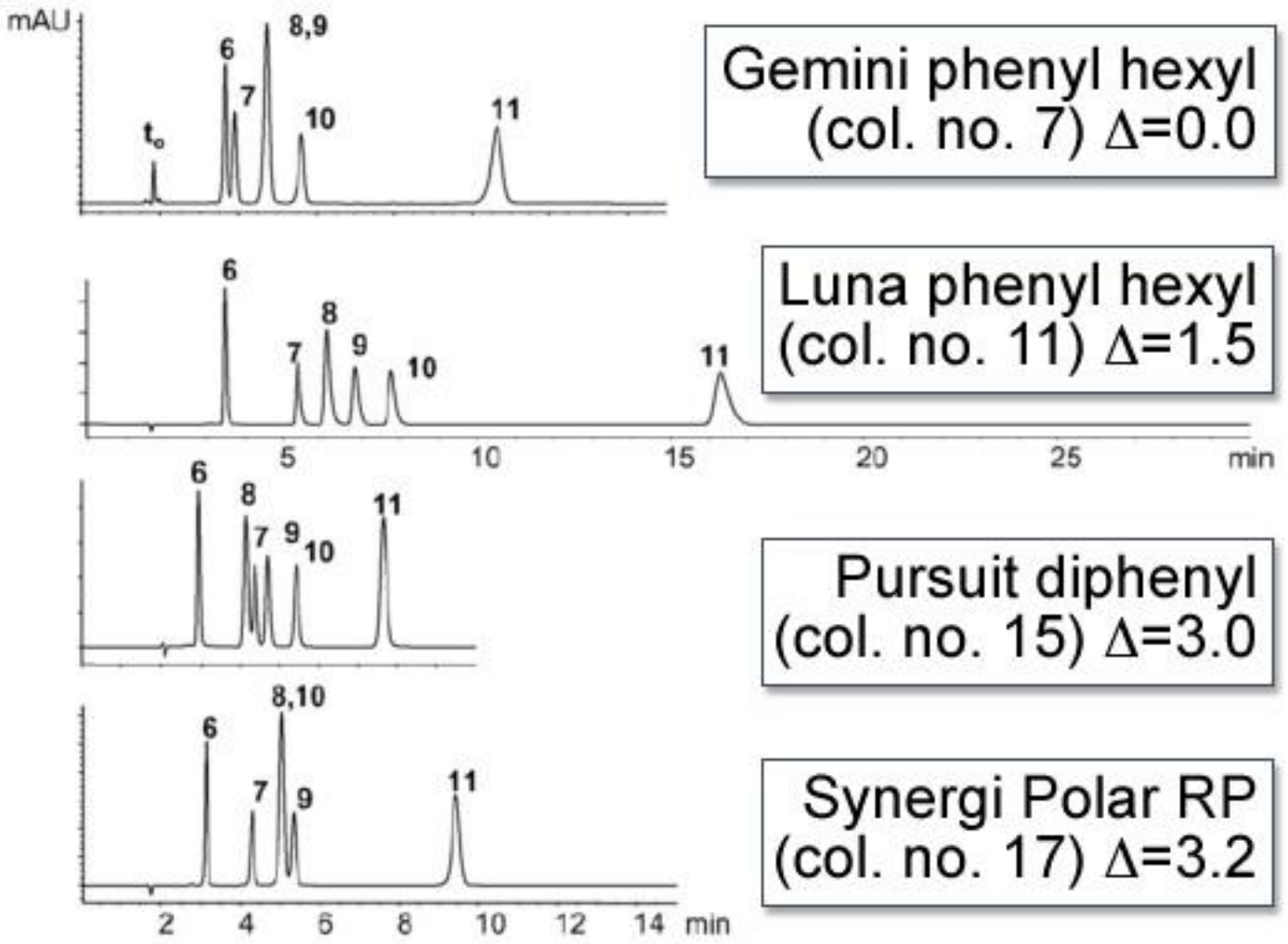
2,4-dinitrotoluene (DNT)



Nitrobenzene (NB)

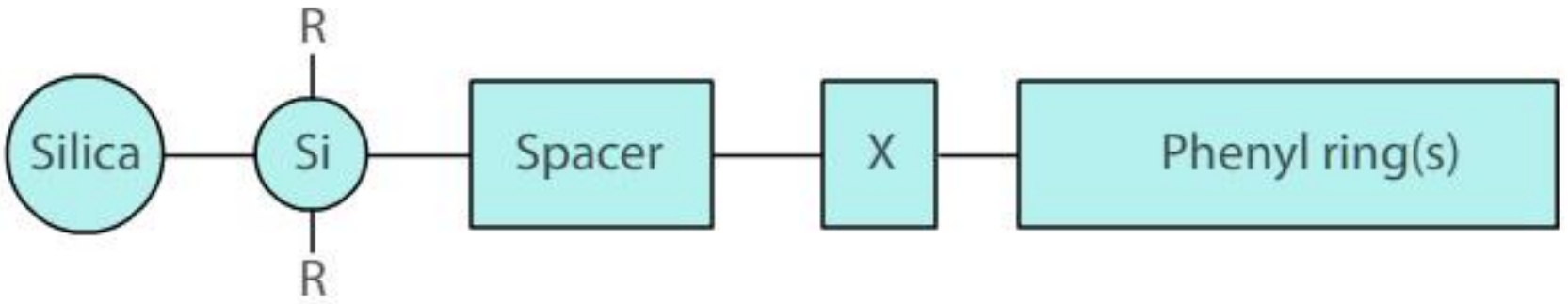
Orthogonality in 'Phenyl' phases

20mM KH_2PO_4, pH 2.7 in MeOH:H_2O (45.5:54.5, v/v), 60 °C, 5µl injection of a lipophilic base test mixture and detection at 210 nm



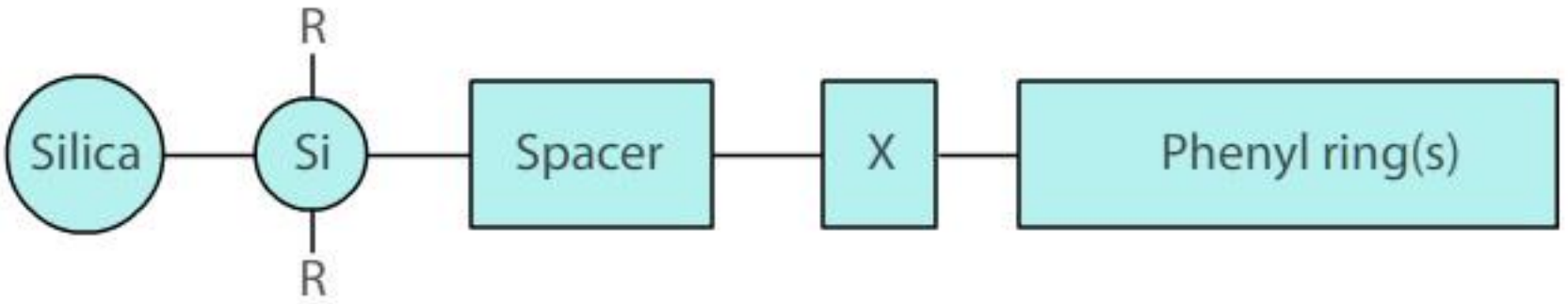
M. R. Euerby, P. Petersson J. Chromatogr. A ***1154*** *(2007)*

Conclusions on Phenyl Phases (I)



- Phenyl phases tend to show lower hydrophobic retention than their C18 counterparts
- The length of the alkyl spacer / inclusion of an electronegative atom strongly influences p-p interaction
- Main difference between phenyl (π-base) and pentafluorophenyl (PFP) phases (π-acid) is enhanced shape selectivity and reduced aromatic selectivity

Conclusions on Phenyl Phases (II)



- Phenyl phases appeared to have a higher hydrogen bonding capacity than their alkly counterparts (although caffeine may notbe an appropriate probe)
- Phenyl phases with longer alkyl linking chains exhibit enhanced hydrophobicity, increased shape and aromatic selectivity, decreased ion exchange and increased apparent hydrogen bonding capacity

USP Characterization Methodology

- The USP Working Group on HPLC columns
- Members of the National Institute of Standards and Technology (NIST) and the five largest manufacturers of HPLC columns in the United States
- This group uses the NIST Standard Reference Material SRM 870 to evaluate columns using the following test conditions

Mobile phase: 80 % methanol / 20 % buffer (v/v) (5 mmol/L potassium phosphate adjusted to pH 7)

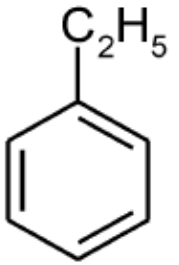
Flow rate: 2 mL/min

Column temp: 23°C ± 2°C

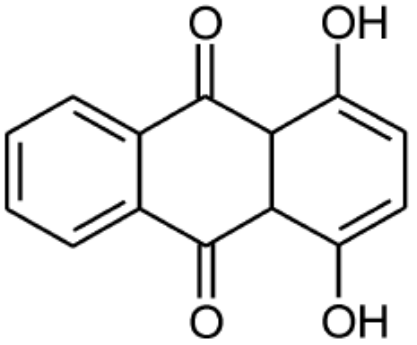
Inj. Vol.: 5 µL

USP Probes

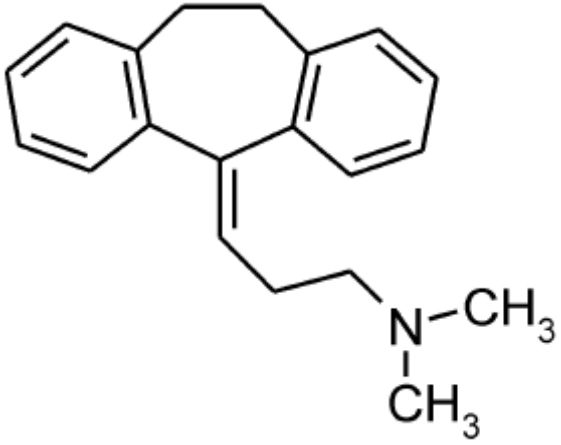
Hydrophobicity / column retentiveness
(capacity factor of ethylbenzene, H or Hy)



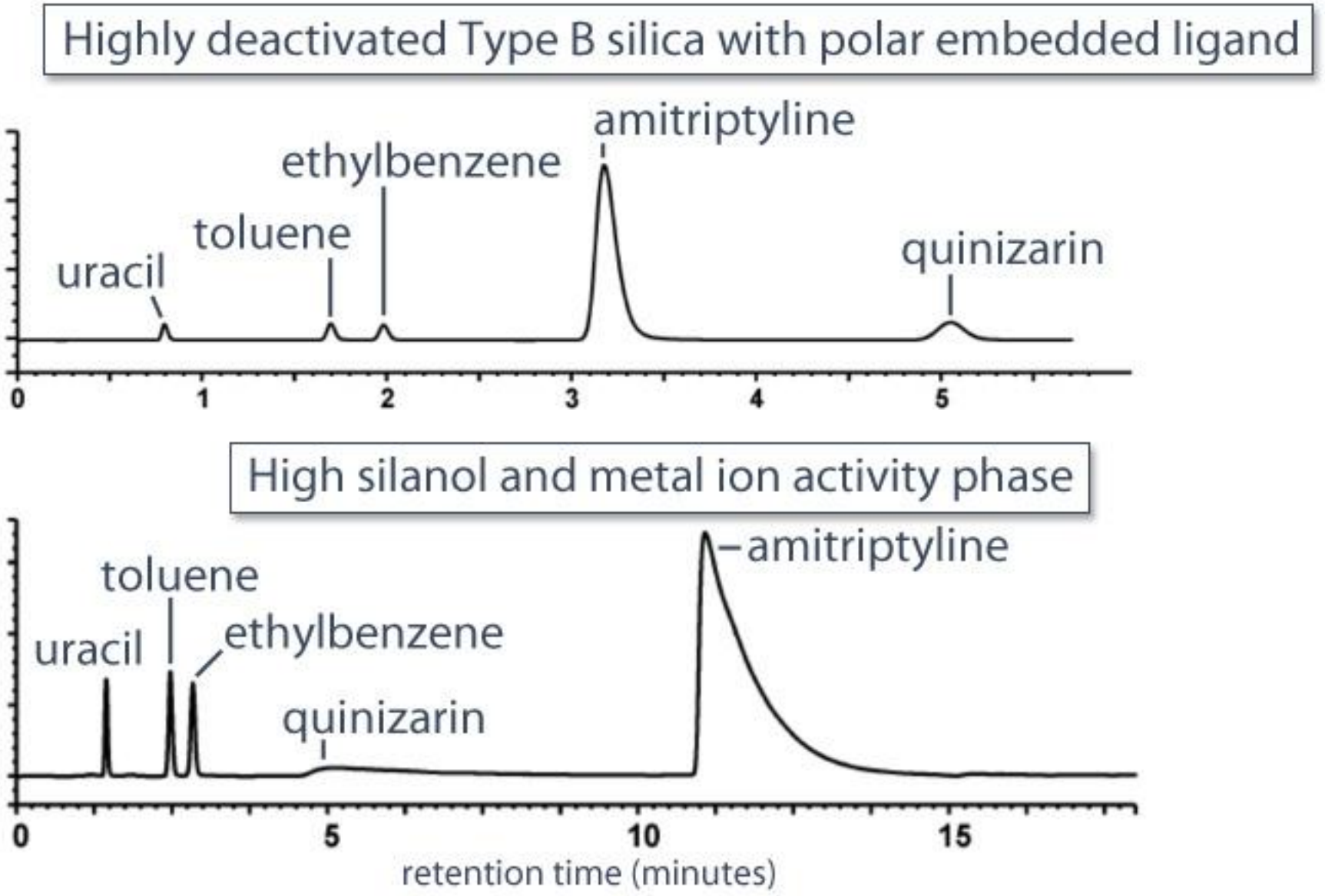
Chelation
(tailing factor of quinizarin, C or CTF)



Activity toward bases (silanol activity)
(capacity factor CA or CTA, and tailing factor of Amitriptyline, TA or TFA)



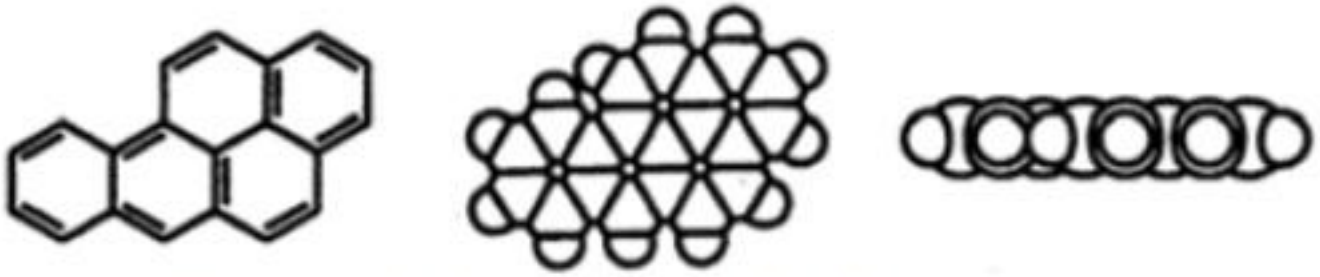
USP – Typical Results



USP – Shape Selectivity

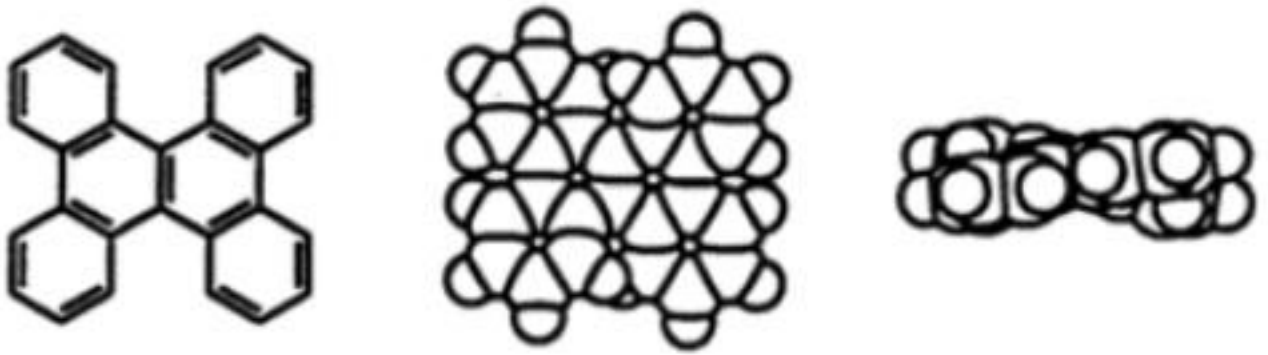
SRM879a

BaP =



Benzo[a]pyrene, BaP

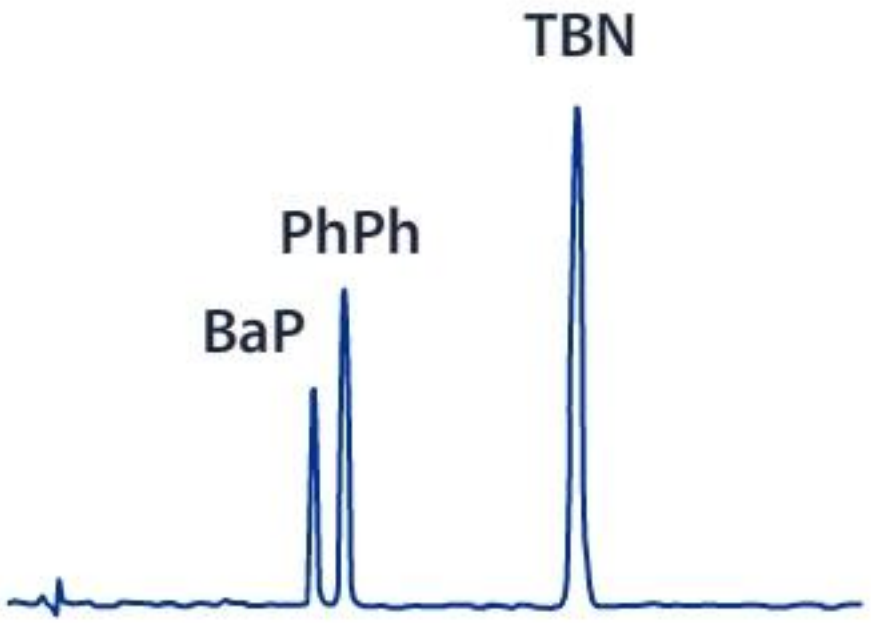
TBN =



1, 2:3, 4:5, 6:7, 8-Tetrabenzonaphthalene, TBH

PhPh = phenanthro[3,4-c]phenanthrene

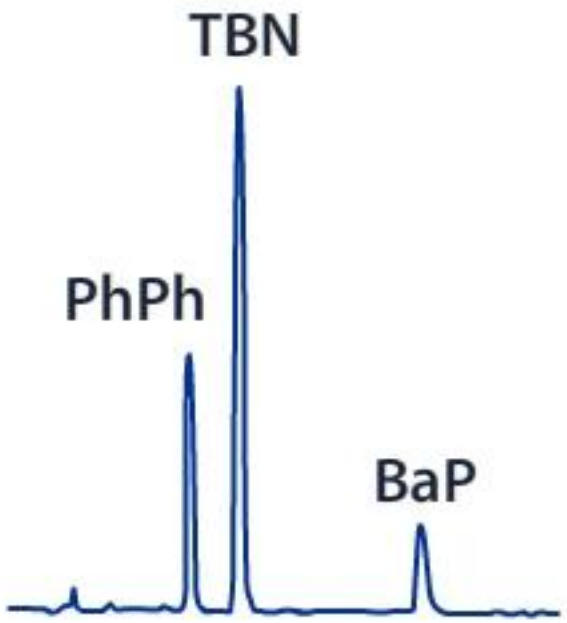
USP – Shape Selectivity



Monomeric C_{18}

$\alpha_{TBN/BaP} > 1.7$

Low shape selectivity



Polymeric C_{18}

$\alpha_{TBN/BaP} < 1$

High shape selectivity

USP – Quantitative Ranking Factor

$$F = \sqrt{\frac{(H_2 - H_1)^2}{VarH} + \frac{(C_2 - C_1)^2}{VarC} + \frac{(CA_2 - CA_1)^2}{VarCA} + \frac{(TA_2 - TA_1)^2}{VarTA} + \frac{(BD_2 - BD_1)^2}{VarBD}}$$

$$BD = \frac{\%C}{100 \times SA \times nC \times 12 \times \left[1 - \left(\frac{\%C \times (MW - 1)}{100 \times nC \times 12}\right)\right]}$$

BD = surface coverage (mol/m^2)
%C = percent carbon loading of the bonded silica
nC = number of carbon atoms in the bonded ligand
MW = molecular weight of the bonded ligand
SA = surface area of silica substrate (m^2/g)

USP – Column Selection Database

HyPurity C18 (Thermo Scientific)

Then select which parameters are more important for your chromatographic procedure:

CTF: ☑ CFA: ☑ TFA: ☑ BD: ☑

The database will automatically display the first 10 columns that, theoretically, could be equivalent to your column. The column with rank 0 is your column. The smaller the F value more similar are the columns, at least theoretically.

Rank	F	Column	Hy	CTF	CFA	TFA	BD	USP Designation	Manufacturer
0	0	**HyPurity C18**	1.3	1.4	3.5	2.1	3.3	L1	Thermo Scientific
1	0.38	**Hypersil BDS 18**	1.5	1.5	3.5	2	3.1	L1	Thermo Scientific
111	6.39	**Supelcosil LC18**	1.3	2	4.5	13	3.1	L1	Supelco

Similar and orthogonal columns to HyPurity C18 according to the USP classification database

PQRI / Hydrophobic Subtraction Model

- The Impurities Working Group of the PQRI Drug Substance Technical Committee with Snyder and Dolan - Hydrophobic Subtraction Model.
- This model is very well characterized and widely reported in literature

Reversed phase conditions:
50% acetonitrile / buffer;
pH 2.8 and 7.0; 35°C

Mixture 1 thiourea amitriptyline 4-butylbenzoic acid	**Mixture 2a** nortriptyline acetophenone mefenamic acid
Mixture 1a *N,N*-diethylacetamide 5-phenyl-1-pentanol ethylbenzene	**Mixture 3** *p*-nitrophenol anisole 4-hexylaniline
Mixture 2 *N,N*-dimethylacetamide 5,5-diphenylhydantoin toluene	**Mixture 3a** *cis/trans* chalcone benzonitrile
	Mixture 4 berberine

N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171-193.

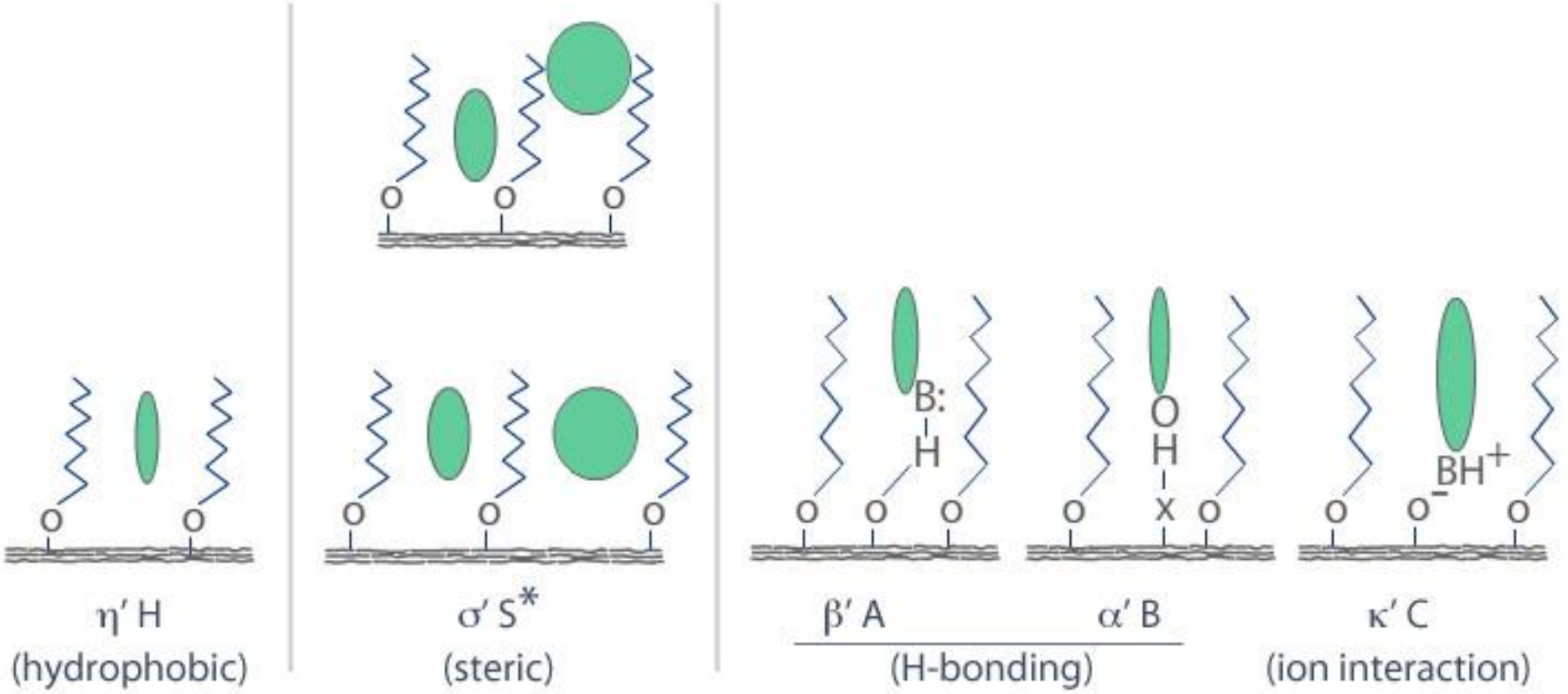
Hydrophobic Subtraction Model

$$\log \alpha = \log k/k_{EB} = \eta'\mathbf{H} - \sigma'\mathbf{S}^* + b'\mathbf{A} + \alpha'\mathbf{B} + \kappa'\mathbf{C}$$

- relative retention (k_{EB})
- hydrophobicity (H)
- steric interaction (S*)
- hydrogen-bond acidity (A) and basicity (B)
- relative silanol ionization or cation-exchange capacity (C) at pH (2.8 and 7.0)

N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002) 171-193.

Hydrophobic Subtraction Model



N.S. Wilson, M.D. Nelson, J.W. Dolan, L.R. Snyder, R.G. Wolcott, P.W. Carr, J. Chromatogr. A 961 (2002).

Hydrophobic Subtraction Model - Quantitative

$$F_s = \sqrt{(H_2 - H_1)^2 + (S_2^* - S_1^*)^2 + (A_2 - A_1)^2 + (B_2 - B_1)^2 + (C_2 - C_1)^2}$$

Fs values below 3 are considered excellent matches
Fs values below 5 are considered reasonable matches
Fs values above 5 are considered poor matches.

Select the option Acids present, if there are acids present in the sample, or Bases present, if there are bases present in the sample. Select the pH of the mobile phase. The default is from 2.8 up to 7.0. pH values outside this range are not going to be accepted.

Acids present: ☐ Bases present: ☑ pH of mobile phase: 2.8 Update

The database will automatically display the first 10 columns that, theoretically, could be equivalent or very different to/from your column, depending on the option you selected. The column with rank 0 is your column. The smaller the F value more similar are the columns, at least theoretically. The higher the F value more different are the columns.

Rank	F	Column	H	S	A	B	C(2.8)	C(7.0)	Type	USP Designation	Manufacturer
0	0	Hypersil GOLD	0.881	0.002	-0.017	0.036	0.162	0.479	B	L1	Thermo/Hypersil
1	1.59	Ultrasphere Octyl	0.896	0.016	0.003	0.086	0.157	0.547	B	L7	Hichrom
1	280.44	EC Nucleosil 100-5 Protect 1	0.544	0.048	-0.411	0.309	-3.213	-0.573	EP		Macherey Nagel

Thank You