Molecular Probes to Characterize HPLC Column Performance

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Use of Chemical Probes in HPLC

- "… the best overall characterization of a reversed-phase bonded phase is done by well-designed chromatographic tests." Uwe Neue, *HPLC Columns*, Wiley (1997)[1]. Probe mixtures have been developed by academic and industrial scientists since about 1990 in attempts to better understand HPLC column separations. Suppliers make use of probes to create columns that separate important classes of compounds. 1.Uwe D. Neue, *HPLC Columns,* Wiley-VCH (1997).
- User groups should consult probe studies published by researchers and column manufacturers extensively during HPLC method development. According to Neue, probes are the best way to identify (or eliminate) columns and mobile phases for selectivity toward target functional groups, and column manufacturers and academicians have best access to a variety of probe molecules. It is important to publish selectivity studies.
- Methanol is a particularly important mobile phase solvent. It should become more popular with users because column selectivity is often different and may be improved over acetonitrile in binary or ternary combinations with water.
- Superficially porous particle (SPP) columns facilitate high sample throughput. More columns and solvent systems can be evaluated to find optimum rather than adequate solutions. Lower pressure than sub-2 µm columns permits use of faster flow rates and higher viscosity methanol/water mobile phases. High efficiency and resolution minimizes the chance of peak overlap.

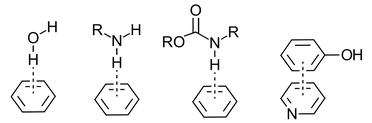
HALO® Reversed-Phase Columns for HPLC and UHPLC

Increasing Polarity (solute dependent)

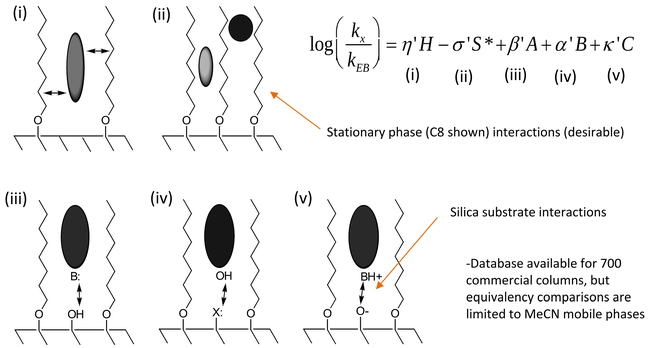
Packing Description	USP Code	Bonded Phase	Types of Interactions
C18	L1	C18 (dimethyloctadecylsilane)	• Hydrophobic
C30	L62	C30 (Triacontyldimethylsilane)	• Hydrophobic
C8	L7	C8 (dimethyloctylsilane)	• Hydrophobic
AQ-C18	L1	proprietary	• Mainly hydrophobic • Some dipole-dipole
Phenyl-Hexyl	L11	Phenyl-Hexyl (dimethylphenylhexylsilane)	• Hydrophobic • π - π
Biphenyl	L11	Biphenyl (dimethylbiphenylsilane)	• π – π • Hydrophobic
RP-Amide	L60	C16 Amide	• Hydrogen Bonding • Hydrophobic
ES-CN	L10	ES-CN (di-isopropylcyanopropylsilane)	• Dipole-dipole • Hydrophobic
PFP	L43	PFP (pentafluorophenylpropylsilane)	• Hydrophobic • π - π • Dipole-dipole • Hydrogen bonding

Aromatic Compounds (with Pi electrons)

* Pi electrons- aromatic systems can be viewed as a large, planar surface with potential for polar interactions, such as Hydrogen Bond accepting ability. Delocalized electrons in the ring system interact with individual polar atoms and other aromatic networks to form strong donor-acceptor complexes that create unique chromatographic retention. Dissociation energies are similar in strength to H-Bonds.



Hydrophobic Subtraction Model[2]



$$\log\left(\frac{k_x}{k_{EB}}\right) = \eta' H - \sigma' S^* + \beta' A + \alpha' B + \kappa' C$$

(i) (ii) (iii) (iv) (v)

-Database available for 700 commercial columns, but equivalency comparisons are limited to MeCN mobile phases

2. Lloyd R. Snyder, Joseph J. Kirkland and John W. Dolan, *Introduction to Modern Liquid Chromatography*, John Wiley and Sons, Inc. (2010).

Orthogonal HALO® Phases: Fs Values for HALO® Phases vs. HALO® C18

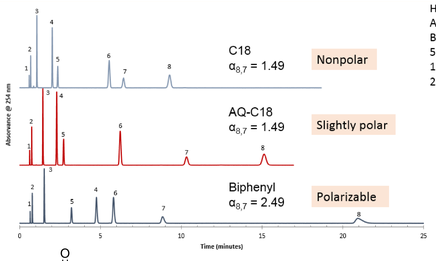
$$F_s = \sqrt{\left(w_H(H_1 - H_2)\right)^2 + \left(w_{S^*}(S^*_1 - S^*_2)\right)^2 + \left(w_A(A_1 - A_2)\right)^2 + \left(w_B(B_1 - B_2)\right)^2 + \left(w_{C_{2.8}}(C_{2.8_1} - C_{2.8_2})\right)^2}$$

F_s	Name	H	S*	A	B	C (pH 2.8)	C (pH 7.0)	ethylbenzene k
0	HALO C18	1.10	0.04	0	-0.05	0.05	0.04	6.1
12.07	HALO AQ-C18	1.00	-0.036	0.099	-0.048	0.156	0.864	6.7
10.04	HALO C8	0.91	0.02	-0.13	0	-0.01	0.18	4.3
17.43	HALO C30	0.938	-0.046	-0.14	0.023	0.17	0.35	4.5
52.83	HALO RP-Amide	0.85	0.08	-0.38	0.19	-0.41	0.31	4.6
17.35	HALO Phenyl-Hexyl	0.780	-0.09	-0.23	0	0.1	0.45	3.5
26.76	HALO Biphenyl	0.708	-0.183	-0.279	0.028	0.047	0.99	3.1
94.45	HALO PFP	0.702	-0.117	-0.073	-0.062	1.17	0.972	2.3
22.78	HALO ES-CN	0.566	-0.11	-0.344	0.021	0.126	1.15	1.88

- $F_s \leq 3$: columns are equivalent
- $3 < F_s < \sim 10$: columns are likely equivalent for samples with a few solutes
- $F_s \geq 35$: columns are orthogonal

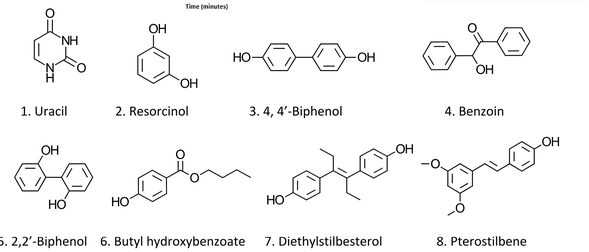
Data provided by Dwight Stoll group at Gustavus Adolphus College. www.hplccolumns.org

Impact of Phase Polarity for OH Probes



HALO 90 Å , 4.6 X 100 mm, 2.7 µm
A=WATER
B= MEOH
55% B ISOCRATIC
1.5 mL/min, 30 °C, 388 bar
25 Hz, 254 nm, 1.0 µL injection

Peak order shown for C18 in MeOH



1. Uracil 2. Resorcinol 3. 4, 4′-Biphenol 4. Benzoin

5. 2,2′-Biphenol 6. Butyl hydroxybenzoate 7. Diethylstilbesterol 8. Pterostilbene

- Retention and selectivity increases for solutes with strong OH dipoles although the stationary phases above are not considered to have H-bonding ability; AQ-C18 has a small amount of dipole character to increase wettability in pure water.
- Aromatic stationary phases, such as Biphenyl, show extra retention and even peak reversal for OH solutes because delocalized electrons are polarizable and more shape selective. Biphenyl is typically more retentive than Phenyl-Hexyl with different selectivity because it has two ring systems and no alkyl character.

Enhanced Selectivity Values for OH Probe Mix in MeOH Mobile Phases

Phase in MeOH	R^2	S Value
HALO® C18	1.0	0
HALO® AQ-C18	0.970	17.3
HALO® C30	0.992	8.9
HALO® C8	0.880	34.6
HALO® Phenyl-Hexyl	0.971	17.0
HALO® Biphenyl	0.872	35.8
HALO® RP-Amide	0.836	40.4

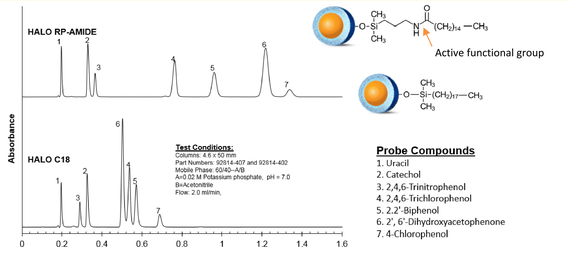
Phase in MeCN	R^2	S Value
HALO® C18	1.0	0
HALO® AQ-C18	0.987	11.8
HALO® C30	0.995	7.1
HALO® C8	0.979	14.5
HALO® Phenyl-Hexyl	0.988	11.0
HALO® Biphenyl	0.968	17.9
HALO® RP-Amide	0.927	50.7

$S = 100\sqrt{(1-R^2)}$

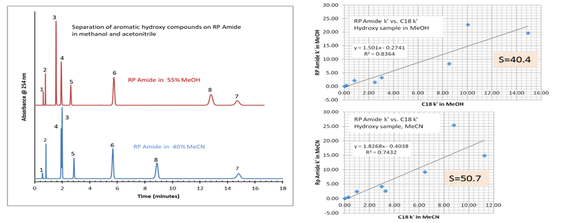
Selectivity difference values by Neue are often enhanced in MeOH although the use of MeCN is more common because of lower operating pressure. One exception to this rule is often RP-Amide.

Using SPP particles, elevated temperatures or ternary MeCN/MeOH blends reduce operating pressure.

Impact of Hydrophobicity Plus Strong Hydrogen Bonding



RP-Amide Shows Different Selectivity in MeOH for OH Probe



- While use of HALO® RP-Amide in MeCN mobile phase is more common, peak spacing and resolution may be improved in MeOH, making it preferred for this sample. Since H-bonding selectivity is different for RP-Amide in the two solvents, a ternary mobile phase may be ideal for difficult separations.

Probe Conclusions/ Acknowledgements

- Methanol is an important HPLC solvent for mobile phase use, but may be overlooked because acetonitrile works so well with C18 columns that are deeply entrenched in HPLC methods. In the interest of saving time, users are tempted to settle for adequate methods on C18 columns using acetonitrile mobile phases.
- Users should screen columns with sample components and the widest range of bonded phases that includes alternatives to hydrophobic interactions and not be limited to C18 and acetonitrile.
- Test probes are clearly the best way to develop a better understanding of how columns and mobile phases work, and skilled users should be more widely encouraged to publish results with probe compounds that are not proprietary.
- Rugged columns with SPP particles are ideal for broad early screening because their fundamental design guarantees fast separation results, moderate pressure, and high peak resolution. Time saved by screening with SPP columns allows study of more phases and variables such as methanol mobile phases. The lower operating pressure and high efficiency of SPP particles also makes SPP columns attractive for the final method.

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