HPLC Method Development Guide

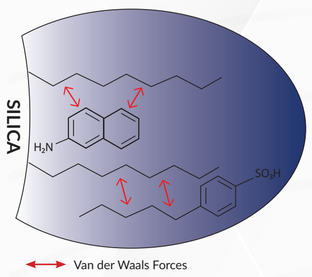
Dr. Maisch
Any Column, Any Size, Any Media

Important Parameters for Reversed-Phase HPLC Method Development

- Structural Formula and pK_a of the Analytes
- Composition of the Mobile Phase
- Selection of the Optimal HPLC Column (Stationary Phase)

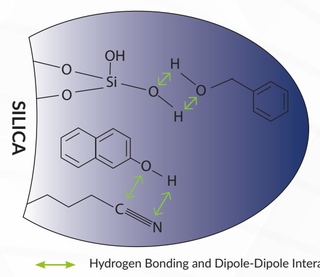
Hydrophobic / Nonpolar / Reversed-Phase (RP)

Typical Stationary Phases: C18, C8, Phenyl, Polymer
Illustration: Interactions with alkyl chains.



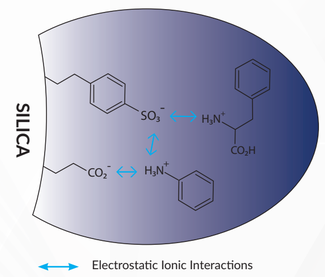
Hydrophilic / Polar / Normal-Phase (NP)

Typical Stationary Phases: Silica, CN, NH_2, HILIC
Illustration: Interactions with silanols and polar functional groups.



Ion Exchange (IEX)

Typical Stationary Phases: SCX, SAX, WCX, NH_2
Illustration: Interactions with functional groups.



TIP:

For aromatic compounds, phenyl phases offer significantly different selectivity compared to alkyl phases (e.g. C8, C18). Based on the interactions between the π-electrons of the phenyl ring in the stationary phase and the double bonds of your analytes, retention times and elution order can change significantly - potentially improving your method.

The selectivity of a phenyl phase can be altered by using methanol instead of acetonitrile as the mobile phase. The π-electrons of acetonitrile can engage in competing interactions with the stationary phase, which may reduce analyte retention.

Mobile Phase – Buffer and Solvent

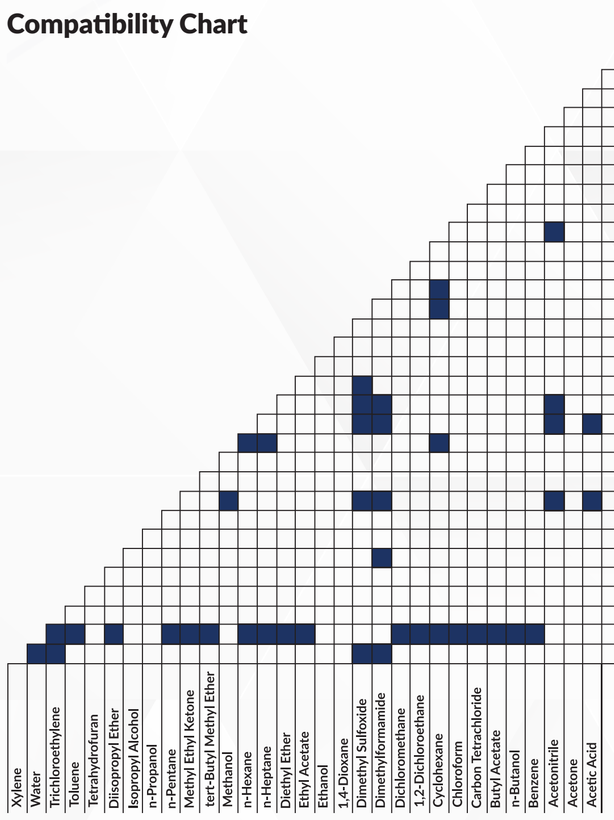
According to Henderson–Hasselbalch:

Use a pH value that is at least two units above or below the analyte's pK_a value. Only then will the substance exist clearly in either the charged or uncharged form.

IMPORTANT!

A buffer always consists of an acid or base and its corresponding salt.
Typical concentrations: 10 – 50 mM, depending on the ionic strength and solubility of the buffer system. Further details can be found in the buffer table below.

Compatibility Chart



Solvent	Polarity Index	Refractive Index @ 20 °C	UV [nm] Cutoff @1 AU	Boiling Point [°C]	Viscosity [cP]	Solubility in Water (% w/w)
Acetic Acid	0.2	1.372	230	118	1.26	100
Acetone	5.1	1.359	330	56	0.32	100
Acetonitrile	5.8	1.344	190	82	0.37	100
Benzene	2.7	1.501	1.501	80	0.65	0.18
n-Butanol	4.0	1.394	1.394	125	0.73	0.43
Butyl Acetate	3.9	1.399	1.399	118	2.98	7.81
Carbon Tetrachloride	1.6	1.466	1.466	77	0.97	0.08
Chloroform	4.1	1.446	1.446	61	0.57	0.815
Cyclohexane	0.2	1.426	1.426	81	1.00	0.01
1,2-Dichloroethane[1]	3.5	1.444	1.444	84	0.79	0.81
Dichloromethane[2]	3.1	1.424	1.424	41	0.44	1.6
Dimethylformamide	6.4	1.431	1.431	155	0.92	100
Dimethyl Sulfoxide[3]	7.2	1.478	1.478	189	2.00	100
1,4-Dioxane	4.8	1.422	1.422	101	1.54	100
Ethanol	5.2	1.360	1.360	78	1.20	100
Ethyl Acetate	4.4	1.372	1.372	77	0.45	8.7
Diethyl Ether	2.8	1.353	1.353	35	0.32	6.89
n-Heptane	0.0	1.387	200	98	0.39	0.0003
n-Hexane	0.0	1.375	200	69	0.33	0.001
Methanol	5.1	1.329	205	65	0.60	100
tert-Butyl Methyl Ether[4]	2.5	1.369	210	55	0.27	4.8
Methyl Ethyl Ketone[5]	4.7	1.379	329	80	0.45	24
n-Pentane	0.0	1.358	200	36	0.23	0.004
n-Propanol	4.0	1.384	210	97	2.27	100
Isopropyl Alcohol[6]	3.9	1.377	210	82	2.30	100
Diisopropyl Ether	2.2	1.368	220	68	0.37	
Tetrahydrofuran	4.0	1.407	215	65	0.55	100
Toluene	2.4	1.496	285	111	0.59	0.051
Trichloroethylene	1.0	1.477	273	87	0.57	0.11
Water	0.0	1.333	200	100	1.00	100
Xylene	2.5	1.500	290	139	0.61	0.018

■ Immiscible
□ Miscible

Immiscible means that in some proportions two phases will be produced.

Synonym Table
[1] Ethylene Chloride
[2] Methylene Chloride
[3] Methyl Sulfoxide
[4] Methyl Tert-Butyl Ether
[5] 2-Butanone
[6] 2-Propanol

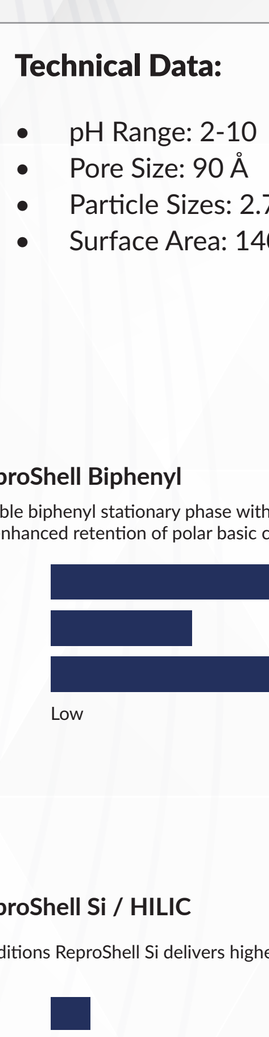
Selection of the Appropriate Buffer

Buffer	pK_a	Buffer Range (pH)	MS Compatible
Trifluoroacetic Acid	< 2	< 2.5	√
Phosphoric Acid (pK_{a1})	2.1	1.1 - 3.1	x
Citric Acid (pK_{a1})	3.1	2.1 - 4.1	x
Formic Acid	3.8	2.8 - 4.8	√
Citric Acid (pK_{a2})	4.7	3.7 - 5.7	x
Acetic Acid	4.8	3.8 - 5.8	√
Citric Acid (pK_{a3})	5.4	4.8 - 6.0	x
Carbonic Acid (pK_{a1})	6.4	5.4 - 7.4	√
Phosphoric Acid (pK_{a2})	7.2	6.2 - 8.2	x
Triethanolamine	7.8	6.8 - 8.8	√
Tris	8.3	7.3 - 9.3	x
Diethanolamine	8.9	7.9 - 9.9	√
Ammonia	9.2	8.2 - 10.2	√
Ethanolamine	9.5	6.5 - 10.5	√
Carbonic Acid (pK_{a2})	10.3	9.3 - 11.3	√
Diethylamine	10.5	9.5 - 11.5	√
Triethylamine	11.0	10.0 - 12.0	x
Piperidine	11.1	10.1 - 12.1	x
Phosphoric Acid (pK_{a3})	12.3	11.3 - 13.3	x

Elution Strength of the Solvents

TIP:

Use both methanol and acetonitrile to vary selectivity.



$$K_a = \frac{[H^+][A^-]}{[HA]}$$

$$pH = pK_a + \log \frac{[A^-]}{[HA]}$$

[HA] / [A-]	pH
99:1	pK_a - 2
9:1	pK_a - 1
1:1	pK_a
1:9	pK_a + 1
1:99	pK_a + 2

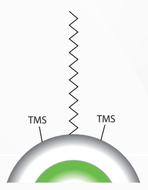
HPLC Stationary Phases – Core-Shell Phases made by Dr. Maisch

Dr. Maisch's **ReproShell** line of core-shell HPLC columns offers high-efficiency chromatographic performance with an innovative superficially porous particle design that delivers sharper peaks, excellent resolution, and lower backpressure compared to fully porous alternatives.

Technical Data:

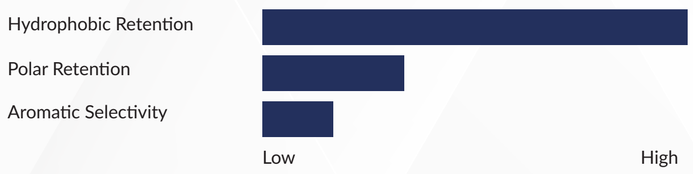
- pH Range: 2-10
- Pore Size: 90 Å
- Particle Sizes: 2.7 µm and 5 µm
- Surface Area: 140 m²/g

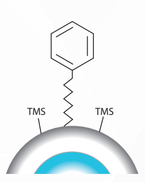
Selection of Stationary Phases:



ReproShell C18 & ODS-1, ODS-3

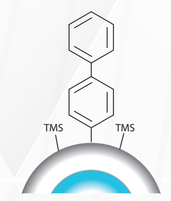
USP L1: All-purpose C18 core-shell column with a well-balanced hydrophobic selectivity and hydrophobic endcapping.





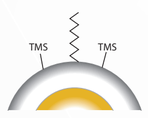
ReproShell Phenyl Hexyl

USP L11: Aromatic and moderate hydrophobic selectivity result in a high retention of aromatic compounds.



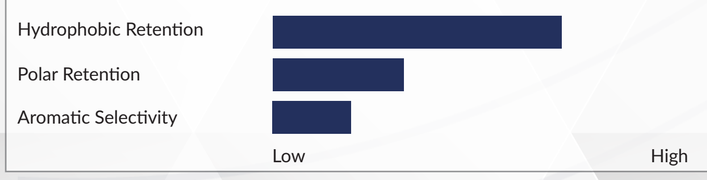
ReproShell Biphenyl

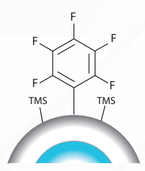
USP L11: 100% water-stable biphenyl stationary phase with an excellent hydrophobic aromatic selectivity plus enhanced retention of polar basic compounds.



ReproShell C8

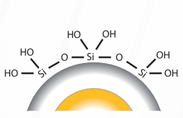
USP L7: Moderate hydrophobic and steric selectivity, ultra-robust and ideal for all methods using octylsilyl stationary phases.





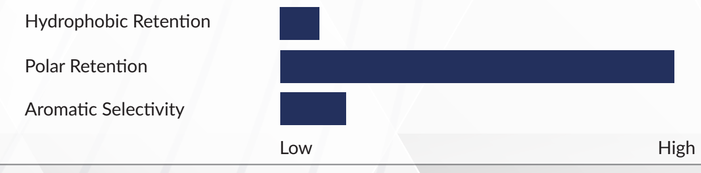
ReproShell PFP

USP L43: Pentafluorophenyl (PFP) stationary phase with a unique mix of hydrophobic, steric, polar and aromatic selectivity.



ReproShell Si / HILIC

USP L3: Under HILIC conditions ReproShell Si delivers highest polar selectivity and excellent separation.



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